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MECANISMOS DE ATEROSCLEROSIS Y ENFERMEDAD CARDIOVASCULAR EN ENFERMEDADES AUTOINMUNES SISTÉMICAS: INTEGRACIÓN DE ANÁLISIS INMUNOLÓGICOS, MOLECULARES Y EPIGENÉTICOS

Carlos Pérez Sánchez

Córdoba, 2017

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Memoria presentada por

Carlos Pérez Sánchez

Licenciado en Biología, para optar al grado de

Doctor en Biología

Tesis doctoral realizada bajo la dirección de las Dras. Rosario López Pedrera y Nuria Barbarroja Puerto en el Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC).

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TÍTULO DE LA TESIS: Mecanismos de aterosclerosis y enfermedad cardiovascular en enfermedades autoinmunes sistémicas: integración de análisis inmunológicos, moleculares y epigenéticos

DOCTORANDO/A: Carlos Pérez Sánchez

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D. Carlos Pérez Sánchez presenta un trabajo original en el que se ha analizado los mecanismos subyacentes al desarrollo de la aterotrombosis en enfermedades autoinmunes sistémicas (Sindrome Antifosfolípido, Lupus Eritematoso Sistemico y Artritis Reumatoide), mediante la integración de análisis inmunológicos, celulares, moleculares y epigeneticos.

La tesis presentada por el doctorando es una tesis por compendio de articulos y mención internacional. Los resultados obtenidos de este trabajo han sido publicados en revistas cientificas de reconocido prestigio internacional en el campo de la investigacion en Reumatología y enfermedad cardiovascular: *Annals of Rheumatic Diseases*, *Arteriosclerosis, Thrombosis and Vascular Biology* y *Scientific Reports*.

La tesis doctoral presentada se enmarca dentro de diversos proyectos de investigación financiados por el Fondo de Investigación Sanitaria (PI12/01511 y PI15/01333) y la Consejería de Innovación, Ciencia y Empresa (CTS-7940) de la Junta de Andalucía.

Finalmente, cabe destacar la formacion técnica y cientifíca alcanzada por el doctorando. El desarrollo de la tesis le ha permitido adquirir conocimientos teóricos y metodológicos que lo capacitan para desarrollar nuevas hipótesis y participar activamente en la redacción y coordinación de nuevos articulos cientificos y proyectos de investigación.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 30 de Marzo de 2017

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TÍTULO DE LA TESIS: Mecanismos de aterosclerosis y enfermedad cardiovascular en enfermedades autoinmunes sistémicas: integración de análisis inmunológicos, moleculares y epigenéticos

DOCTORANDO/A: Carlos Pérez Sánchez

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D. Carlos Pérez Sánchez presenta un trabajo original en el que se ha analizado los mecanismos subyacentes al desarrollo de la aterotrombosis en enfermedades autoinmunes sistémicas (Síndrome Antifosfolipido, Lupus Eritematoso Sistémico y Artritis Reumatoide), mediante la integración de análisis inmunológicos, celulares, moleculares y epigeneticos.

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Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 30 de Marzo de 2017

Firma del responsable de línea de investigación

Fdo.: Rosario López Pedrera

TÍTULO DE LA TESIS: Mecanismos de aterosclerosis y enfermedad cardiovascular en enfermedades autoinmunes sistémicas: integración de análisis inmunológicos, moleculares y epigenéticos

DOCTORANDO/A: Carlos Pérez Sánchez

INFORME RAZONADO DEL TUTOR DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El estudio que se presenta en esta tesis doctoral indica que el análisis integrado de los procesos autoinmunes, moleculares, genéticos y epigenéticos, permiten la identificación y caracterización de nuevos biomarcadores de desarrollo de patología vascular aterotrombótica en enfermedades autoinmunes sistémicas: Sindrome Antifosfolipido, Lupus Eritematoso Sistemico y Artritis Reumatoide.

Su desarrollo ha dado lugar a diversas publicaciones en revistas de alto indice de impacto. El compendio de tres de dichas publicaciones constituye el centro de la presente tesis doctoral.

Asimismo, los estudios realizados han sido presentados en varios congresos nacionales (Congreso de la Sociedad Española de Reumatología) e internacionales (congreso de la *European League against Rheumatism*-EULAR- y *American College of Rheumatology* –ACR-) y han sido objeto de varios premios como el "*premio a la publicacion cientifica mas relevante en colaboracion con grupos internacionales*" en la V leccion conmemorativa Maimonides y premios IMIBIC y "*mejor comunicación oral*" de la V jornada de jovenes investigadores del IMIBIC.

Por tanto, el trabajo presentado reúne, a mi juicio, los méritos suficientes para optar al grado de Doctor.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, a 30 de Marzo de 2017

Firma del tutor de la tesis

ABREVIATURAS

Abreviaturas

AAF: Anticuerpos antifosfolípido
aCL: Anticuerpos anticardiolipina
ADN: Ácido desoxirribonucleico
Anti-CCPs: Anticuerpos antipéptidos cíclicos citrulinados
Anti-dsDNA: Anticuerpos contra ADN de doble cadena
Anx I: Anexina I
AR: Artritis reumatoide
ARNm: Ácido ribonucleico mensajero
AT: Aterotrombosis
ATP: Adenosina trifosfato
CAT: Catalasa
CV: Cardiovascular
DAS28: Índice de actividad de enfermedad para Artritis Reumatoide
DNMTs: ADN metiltransferasas
EAS: Enfermedades autoinmunes sistémicas
ECV: Enfermedad cardiovascular
ERK: Quinasa activada por señales extracelulares
ERNs: Especies reactivas de nitrógeno
EROs: Especies reactivas de oxígeno
Factor XI: Factor de coagulación XI
Flt1: Receptor 1 del VEGF
FR: Factor reumatoide
GPx: Glutatión peroxidasa
GR: Glutatión reductasa
GSH: Glutatión reducido
GSSG: Glutatión oxidado
HATs: Enzimas acetil transferasas
HDACs: Enzimas desacetilasas
HDL: Lipoproteínas de alta densidad
HLA: Antígeno leucocitario humano
Hsp60: Proteína de choque térmico 60
ICAM-1: Molécula de adhesión intracelular-1
IFN- γ: Interferón- γ
IgG: Inmunoglobulina G

IgM: Inmunoglobulina M
IL-1: Interleuquina-1
IL-2: Interleuquina-2
IL-6: Interleuquina-6
IL10: Interleuquina-10
IL-17: Interleuquina-17
IMC: Íntima media carotídea
K+: Potasio
LDLox: Lipoptroteínas de baja densidad oxidadas
LES: Lupus eritematoso sistémico
MAPK: Proteínas quinasas activadas por mitógenos
MEK: Quinasa de las MAPK
miRNAs: MicroRNAs
MMP: Metaloproteinasas
NADPHox: Nicotinamida adenina dinucleótido fosfato-oxidasa
NETs: Redes extracelulares de neutrófilos
NF-kappaB: Factor de transcripción-kB
p38 MAPK: p38- proteínas quinasas activadas por mitógenos
PARs: Receptores activados por proteasas
PDI: Proteína disulfuro isomerasa
RISC: Complejo de silenciamiento inducido por ARN
RNA pol: RNA polimerasa
SAF: Síndrome antifosfolípido primario
SLEDAI: Índice de actividad de la enfermedad para LES
SOD: Superóxido dismutasa
TF: Factor tisular
TFPI: Inhibidor de la vía del factor tisular
TNF-α: Factor de necrosis tumoral-α
Treg: Células T reguladoras
UTR: Regiones no traducidas de los genes
VCAM-1: Molécula de adhesión celular vascular-1
VEGF: Factor de crecimiento vascular endotelial
β2-GPI: β2-glicoproteína I

ÍNDICE

RESUMEN

Resumen

Las enfermedades autoinmunes son el resultado de una interacción entre factores genéticos predisponentes, una alteración del sistema inmune y factores ambientales desencadenantes. Numerosos estudios epidemiológicos han demostrado que patologías autoinmunes sistémicas como la Artritis Reumatoide (AR), el Síndrome Antifosfolípido (SAF), y el Lupus Eritematoso Sistémico (LES), presentan una elevada incidencia de eventos cardiovasculares y aterosclerosis y, como consecuencia, altos ratios de mortalidad. El desarrollo de enfermedad cardiovascular en estas patologías implica factores genéticos, así como otros factores de riesgo adquiridos. Los componentes inflamatorios de la respuesta inmune, el estrés oxidativo, así como elementos autoinmunes (auto anticuerpos, auto antígenos y linfocitos auto reactivos) parecen estar también involucrados en estos procesos.

Estudios recientes indican que los miRNAs desempeñan un papel muy relevante y novedoso en la regulación epigenética de la expresión de genes y proteínas clave en la patología cardiovascular. Por tanto, la identificación y caracterización de los miRNAs asociados a la aterotrombosis en patologías autoinmunes podría contribuir a la identificación de biomarcadores para delinear el fenotipo de dicha patología, así como la valoración de respuesta a nuevas modalidades terapéuticas.

El **Objetivo principal** de esta tesis ha sido el estudio de los mecanismos subyacentes al desarrollo de la aterotrombosis en enfermedades autoinmunes sistémicas (SAF, LES y AR), mediante la integración de análisis inmunológicos, celulares, moleculares y epigenéticos.

Los resultados obtenidos sugieren que:

1. Los anticuerpos anti-CCPs son mediadores clave en el desarrollo del perfil inflamatorio y proaterogénico de los pacientes con AR. Sus efectos son específicos de la diana celular sobre la que actúan, promoviendo un incremento en la expresión de marcadores trombóticos, inflamatorios y prooxidativos en monocitos, un estatus prooxidativo en neutrófilos y un perfil proinflamatorio en linfocitos. Por tanto, los anticuerpos anti-CCPs podrían ser considerados dianas terapéuticas para la prevención del desarrollo de enfermedad cardiovascular en pacientes con AR.

2. El perfil de expresión génica permite la segregación del Síndrome Antifosfolípido primario, el Lupus Eritematoso Sistémico y el Síndrome Antifosfolípido asociado a Lupus, con características específicas que explican los cambios pro-trombóticos, inflamatorios y pro-ateroscleróticos observados en estas enfermedades autoinmunes.

En su conjunto, los resultados de este estudio sugieren que la identificación de genes clave que regulan vías fisiopatológicas específicas permitiría el desarrollo de terapias dirigidas para cada condición autoimmune.

3. Diversos miRNAS, y sus proteínas de biogénesis se encuentran diferencialmente expresados en monocitos y neutrófilos de pacientes con Síndrome Antifosfolípido y Lupus Eritematoso Sistémico, y son modulados por acción de los anticuerpos característicos de ambas patologías. Dichos miRNAs podrían considerarse posibles biomarcadores de la patología aterotrombótica en ambas enfermedades autoinmunes.

En suma, los resultados de los estudios presentados indican que el análisis conjunto de los procesos autoinmunes, moleculares, genéticos y epigenéticos, permiten la identificación y caracterización de nuevos biomarcadores de desarrollo de la patología vascular aterotrombótica en enfermedades autoinmunes sistémicas.

ABSTRACT

Abstract

It is widely known that autoimmune diseases (AIDs) are the result of complex interactions between predisposing genetic factors, deregulation of the immune system, and environmental triggering factors. Several systemic autoimmune conditions, including Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), and Antiphospholipid Syndrome (APS), are linked to enhanced atherosclerosis, and consequently higher cardiovascular morbidity and mortality rates. The development of cardiovascular disease in these pathologies involves genetic factors as well as other acquired and modifiable risk factors (e.g., hypercholesterolemia, diabetes mellitus, and hypertension). Inflammatory components of the immune response, as well as autoimmune elements (e.g., autoantibodies, autoantigens, and autoreactive lymphocytes), seem to be also involved in these processes.

Recent studies indicate that miRNAs play a relevant and novel role in the epigenetic regulation of genes and proteins associated to cardiovascular diseases. Thus, the identification and characterization of miRNAs involved in the control of relevant mediators associated to atherothrombosis in autoimmune diseases could help in the identification of new biomarkers of these disorders.

Based on these premises, the main objective of this thesis was the study of the mechanisms of atherothrombosis in systemic autoinmmune diseases (antiphospholipid syndrome, systemic lupus erythematosus, rheumatoid arthritis), by integrating immunological, molecular, and epigenetic approaches that might contribute to identify new biomarkers of disease eligible for being modulated using new therapeutic strategies.

The results of these studies suggested that:

1. Anti-CCPs are key players in the inflammatory and pro-atherogenic status of RA patients. The effects are specific of the immune cell targeted, promoting over-expression of thrombotic, inflammatory and pro-oxidative markers in monocytes, pro-oxidative status in neutrophils, and pro-inflammatory profile in lymphocytes. Targeting these autoantibodies would be an excellent strategy to prevent the development of cardiovascular disease in RA.

2. Gene expression profiling allows the segregation of APS, SLE and APS plus SLE, with specific signatures explaining the pro-atherosclerotic and pro-thrombotic alterations in these highly related autoimmune diseases. Thus, the identification of key genes that regulate specific pathophysiological pathways would allow the development of targeted therapies for each autoimmune condition.

3. Specific miRNAs might act as potential biomarkers of immune activation and atherothrombosis in APS and SLE patients. Besides, miRNA biogenesis is significantly altered in neutrophils of APS and SLE patients and associated to both, the presence of a pathologic carotid intima-media thickness, and the occurrence of thrombotic events. Anti-dsDNA and antiphospholipid antibodies regulate cardiovascular disease in APS and SLE, at least partially, by modulating the biogenesis and the expression of miRNAs.

Taken together, the results of the presented studies indicate that the integrated analysis of the autoimmune, molecular, genetic and epigenetic processes, allow the identification and characterization of new biomarkers of atherothrombotic vascular pathology development in AIDs.

INTRODUCCIÓN

1. Enfermedad cardiovascular en patologías autoinmunes sistémicas: Lupus Eritematoso Sistémico, Síndrome Antifosfolípido y Artritis Reumatoide.

La enfermedad vascular aterotrombótica representa un problema global de salud pública de creciente importancia. A pesar de los avances que se han realizado en el tratamiento de las manifestaciones clínicas de esta enfermedad (infarto de miocardio, accidentes cerebrovasculares, aneurisma aórtico, vasculopatía periférica, etc.) las recidivas son frecuentes y la morbimortalidad continúa siendo elevada. Un aspecto importante de la prevención y tratamiento de la aterotrombosis (AT) consiste en identificar precozmente a los sujetos con riesgo cardiovascular aumentado, susceptibles de desarrollar episodios trombóticos.

Los nuevos conceptos relacionados con la patogenia de la AT, donde la inflamación generada por un mecanismo inmunológico se considera determinante, han permitido considerar que un proceso autoinmune esté involucrado en su inicio y perpetuación [1]. Las enfermedades autoinmunes sistémicas (EAS) más estudiadas en relación con la AT son aquellas en las que ocurre una mayor incidencia, como el lupus eritematoso sistémico (LES), el síndrome antifosfolípido primario (SAF) y la artritis reumatoide (AR).

El **LES** es una enfermedad autoinmune, multisistémica, crónica, con un amplio rango de manifestaciones clínicas, incluyendo fotosensibilidad, lesiones discoidales, artritis/artralgia, nefritis, enfermedad cardiaca (8-16%), enfermedad pulmonar y desórdenes en el sistema nervioso central. Diversos estudios han demostrado que los pacientes con LES presentan

un riesgo incrementado de desarrollar enfermedad cardiovascular [2-4], siendo esta una de las principales causas de mortalidad [5].

El **SAF** es un desorden autoinmune caracterizado por la asociación de trombosis arteriales y/o venosas y/o una historia obstétrica definida por pérdidas fetales o prematuridad y pre-eclampsia en presencia de anticuerpos antifosfolípido [6]. La presencia simultánea de ambas patologías se define como **síndrome antifosfolípido asociado a lupus** (SAF más LES), en el que se engloban pacientes LES que han sufrido eventos trombóticos o pérdidas fetales en presencia de anticuerpos antifosfolípido.

La **AR** es una enfermedad inflamatoria, autoinmune y crónica de etiología desconocida, que afecta principalmente a grandes y pequeñas articulaciones de forma simétrica, produciendo engrosamiento sinovial, destrucción del cartílago articular y desarrollo de erosiones óseas que conducen a una deformidad articular permanente. Junto a estas complicaciones clínicas y fruto de la inflamación sistémica crónica, aparecen otras afectaciones como la enfermedad cardiovascular (ECV). Diversos trabajos han señalado que los pacientes con AR presentan entre 2 y 3 veces más riesgo de sufrir eventos cardiovasculares que la población general, los cuales repercuten en un aumento de los índices de mortalidad de la enfermedad [7, 8].

El desarrollo de ECV en estas patologías autoinmunes implica factores genéticos y epigenéticos, así como otros factores de riesgo adquiridos (p. ej. hipercolesterolemia, diabetes mellitus e hipertensión). Los componentes protrombóticos e inflamatorios de la respuesta inmune (principalmente el factor tisular y diversas citoquinas), el estrés oxidativo,

así como elementos autoinmunes (p. ej. Auto-anticuerpos, auto-antígenos y linfocitos autorreactivos) parecen estar también implicados en estos procesos [9] (Figura 1).

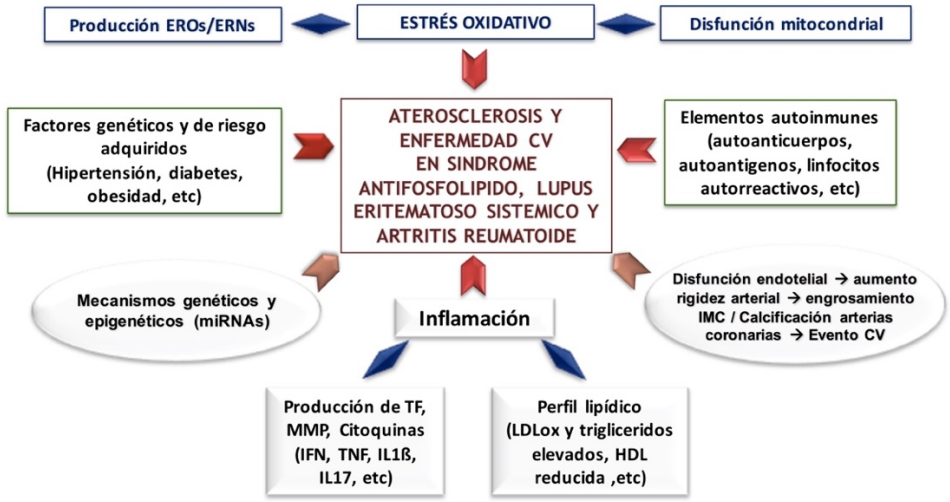

Figura 1. Mecanismos de Aterotrombosis en Síndrome Antifosfolípido, Lupus Eritematoso Sistémico y Artritis Reumatoide. Abreviaturas: EROs, especies reactivas de oxígeno; ERNs, especies reactivas de nitrógeno; IMC, íntima media carotídea; CV, cardiovascular; miRNAs, microRNAs; TF, factor tisular; MMP, metaloproteinasas; LDLox, lipoptroteínas de baja densidad oxidadas; HDL, lipoproteínas de alta densidad.

2. Auto-anticuerpos como factores de riesgo aterotrombótico en patologías autoinmunes

Numerosas evidencias sugieren que la autoinmunidad humoral podría jugar un papel relevante en el desarrollo de ECV, y que ciertos auto-anticuerpos podrían actuar como biomarcadores específicos de riesgo cardiovascular.

2.1 Anticuerpos Antifosfolípido

La activación monocítica inducida por los anticuerpos antifosfolípido (AAF) presentes en pacientes con SAF y en un alto porcentaje de LES, y

que engloban principalmente a anticuerpos anticardiolipina (aCL), anticuerpos anti-β2-glicoproteína (β2-GPI), anticuerpos anti-protrombina y anticoagulante lúpico, se produce por una compleja interacción entre numerosos efectores intracelulares, responsables últimos del desarrollo de trombosis. Hace algunos años Lopez-Pedrera y colaboradores, demostraron que un proceso esencial es la inducción de la actividad procoagulante, mediante la activación del factor tisular (TF), el principal iniciador de la coagulación sanguínea [10]. La señalización intracelular asociada a dicha activación esta mediada por los receptores activados por proteasas (PAR, mediadores de respuestas críticas para la trombosis, hemostasia y procesos inflamatorios y participantes en el desarrollo de arteriosclerosis), cuya expresión se encuentra incrementada en los monocitos de los pacientes con SAF [11]. Estudios complementarios han demostrado que dicha señalización intracelular, inducida por los AAF, implica la activación constitutiva de MAPK y NFkappaB [12], así como la inducción de la expresión del factor de crecimiento vascular endotelial (VEGF) y su receptor Flt1 en monocitos de estos pacientes [13]. De modo adicional, estudios proteómicos posteriores han conducido a la identificación de nuevas proteínas (cuya expresión se encuentra alterada por efecto directo de los AAF) implicadas en el desarrollo de trombosis y la activación de la respuesta inmune (p. ej., protein disulfuro isomerasa, anexina I, anexina II, catalasa, ubiquitin-Nedd8, proteinas RhoA y Hsp60) [14]. La imbricación de todas estas proteínas y mecanismos intracelulares resulta pues en la inducción de un estado procoagulante y proinflamatorio en estos pacientes (Figura 2).

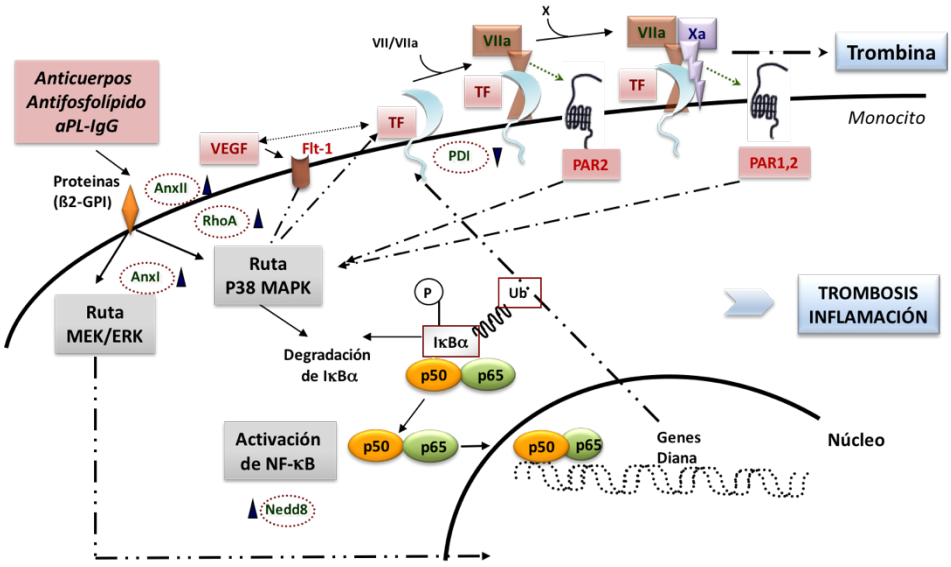

Figura 2. Mecanismos protrombóticos/proinflamatorios en el Síndrome Antifosfolípido inducidos por los anticuerpos antifosfolípido. Abreviaturas: AAF, anticuerpo antifosfolípido; AnxI, anexina I; β2-GPI, β2 glicoproteína I; ERK, quinasa activada por señales extracelulares (del inglés *extracellular signal-regulated kinase*); Flt1, receptor 1 del VEGF (del inglés *fms-like tyrosine kinase-1*); MEK, quinasa de las MAPK; NF-κB, factor de transcripción κB (del inglés *nuclear factor kappa-light-chain-enhancer of activated B cells*); p38 MAPK, p38- proteínas quinasas activadas por mitógenos (del inglés p38-*mitogen activated protein kinases*); PARs, receptores activados por proteasas (del inglés *protease-activated receptors*); PDI, proteína disulfuro isomerasa (del inglés *protein disulfide isomerase*); TF, factor tisular (del inglés *tissue factor*); VEGF, factor de crecimiento endotelial vascular (del inglés *vascular endothelial growth factor*).

Los anticuerpos antifosfolípido y otros autoanticuerpos presentes en SAF (como los anti-HDL o lipoproteínas de alta densidad y anti-apolipoproteina I), son también responsables del desarrollo de enfermedad cardiovascular y aterosclerosis a través de otros mecanismos complementarios, que incluyen la formación de complejos anti-LDLox/anti-ß2GPI, lo que facilita o acelera la absorción de LDLox (lipoproteínas de baja densidad oxidadas) por los macrófagos y su consecuente conversión en células espumosas. Además, tanto macrófagos como células endoteliales, adhieren ß2GPI durante el proceso aterosclerótico, de modo que los anticuerpos promueven la adhesión de los macrófagos al endotelio mediada por moléculas de adhesión como ICAM-1, VCAM-1 o E-selectina [15]. De hecho,

se ha demostrado una correlación positiva entre los títulos de anticuerpos anticardiolipina y anti-ß2GPI-1 y la incidencia y severidad del síndrome coronario agudo, el infarto de miocardio o el accidente cerebrovascular [16].

En un estudio reciente, en una cohorte de 43 pacientes con SAF [17], se ha observado, una correlación positiva entre los títulos de aCL y la ocurrencia de eventos trombóticos en estos pacientes. También se halló una asociación positiva significativa entre los títulos de estos anticuerpos y la presencia de aterosclerosis precoz, medida como engrosamiento de la íntima media carotidea. Estos resultados confirman estudios previos desarrollados por el grupo de Ames *et al*. [18], que también demostraron un aumento en el engrosamiento de la íntima media carotidea asociada al título de aCL de isotipo IgG. Es más, estos datos apuntan a la existencia de una aterosclerosis prematura asociada al desarrollo de trombosis, de forma que, en esta serie de pacientes, 11 de los 12 pacientes SAF que mostraban placas ateroscleróticas habían sufrido al menos un evento trombótico previo.

La secuencia de inicio de formación de la placa aterosclerótica en estos pacientes implicaría: la activación de las células endoteliales por efecto de los anticuerpos aCL y la expresión de moléculas de adhesión que favorecería el reclutamiento de monocitos y linfocitos. Los monocitos activados migran hacia la íntima arterial, donde por acción de los complejos ß2GPI/LDLox se incrementaría la absorción de LDLox, conduciendo a la formación de células espumosas. Estas células, junto con los linfocitos T, producirían citoquinas, factores de crecimiento, TF, etc., promoviendo la proliferación de las células musculares lisas, potenciando así el proceso aterotrombótico.

Aunque se ha demostrado una relación directa entre la presencia de AAF y el desarrollo de AT y ECV en pacientes con SAF+LES, un porcentaje elevado de pacientes lúpicos no presenta positividad para anticuerpos AAF (i.e. aCL o anti-ß2GPI). No obstante, estos pacientes también son susceptibles de sufrir eventos CV.

2.2 Anticuerpos Antinucleares y anti-dsDNA

El distintivo inmunológico de los pacientes lúpicos son los auto-anticuerpos contra proteínas nucleares (ANA) y ADN de doble cadena (anti-dsDNA), principalmente los anticuerpos anti-dsDNA y los inmunocomplejos circulantes dsDNA/anti-dsDNA [19]. Los anticuerpos anti-dsDNA son los más frecuentes y específicos del LES, de forma que sus niveles séricos se utilizan en la clínica para monitorizar la progresión de la enfermedad y la respuesta terapéutica.

En pacientes lúpicos se ha demostrado una correlación de la actividad de la enfermedad con los títulos de anti-dsDNA, adscribiendo así un papel patogénico a dichos anticuerpos [20]. En una cohorte de 85 pacientes, Ruiz-Limón y colaboradores han demostrado recientemente una correlación significativa entre los niveles de anti-dsDNA, la actividad de la enfermedad (SLEDAI) y los niveles séricos de diversos marcadores inflamatorios [21].

Parte de su patogenicidad puede deberse a su capacidad hidrolítica y citotóxica sobre las células de diferentes tejidos y órganos que se ven afectados en el desarrollo de la enfermedad. Además, estos anticuerpos son capaces de interaccionar a nivel de tejido con la α-actinina del glomérulo del riñón, contribuyendo al desarrollo de enfermedad renal [19].

Se ha sugerido asimismo que el dsDNA junto a sus autoanticuerpos induce la producción de IL-1 en monocitos, activando al inflamasoma NLRP3 a través de la síntesis de especies reactivas de oxigeno (EROs) y el eflujo de K+ [22]. Un trabajo realizado en modelos animales y humanos ha demostrado igualmente la contribución de los anti-dsDNA a la patogénesis de la aterosclerosis [23]. En dichos estudios se demuestra que estos anticuerpos promueven la acumulación de colesterol en macrófagos y células musculares lisas, así como la dislipoproteinemia, la citotoxicidad y la liberación de citoquinas.

2.3 Anticuerpos antipéptidos cíclicos citrulinados

Entre los autoanticuerpos detectados en la AR, los anticuerpos antipéptidos cíclicos citrulinados (anti-CCPs) son los más específicos para la enfermedad [24], ya que el otro tipo de autoanticuerpos presente en esta patología autoinmune, el factor reumatoide (FR), de isotipo Ig M, también está presente en un elevado porcentaje de pacientes con otras infecciones crónicas severas. Además, el estatus aterosclerótico en la AR es independiente de los niveles de FR [25]. La detección de los anticuerpos anti-CCPs se ha convertido en una herramienta útil para el diagnóstico de la AR, particularmente en etapas tempranas de la enfermedad [26]. Así, en los nuevos criterios de clasificación de la AR de 2010 se incluyó la detección de los anti-CCPs como un parámetro clave para el diagnóstico de la enfermedad [27]. También se ha sugerido que los niveles de anti-CCPs pueden actuar como predictores de un perfil cardiovascular adverso en pacientes con RA [28,29]. Sin embargo, esta hipótesis surge de estudios de asociación realizados en diferentes cohortes, mientras que ningún estudio ha demostrado un efecto directo de los anti-CCPs en todos esos procesos.

En suma, todos estos datos sugieren la implicación de los auto-anticuerpos en el desarrollo de aterotrombosis en SAF, LES y AR. Sin embargo, aún es necesario analizar en detalle qué auto-anticuerpos y a qué nivel contribuyen al desarrollo de la patología CV en cada condición autoinmune, así como los mecanismos moleculares subyacentes.

3. Las células del sistema inmune como protagonistas del proceso aterotrombótico

Diversos tipos celulares del sistema inmune actúan como elementos clave en el desarrollo de ECV en SAF, LES y AR. La activación e interacción de estas células conduce a la producción de mediadores responsables de la inflamación y la aterotrombosis.

3.1 Monocitos/Macrófagos

Los monocitos/macrófagos juegan un papel esencial en la patogenia de la aterotrombosis. Son, no obstante, un grupo celular heterogéneo, con funciones divergentes en inmunidad, aterogénesis y procesos reparativos. Así, en respuesta a estímulos específicos pueden promover la fagocitosis de lipoproteínas citotóxicas, la eliminación de cuerpos apoptóticos, la secreción de citoquinas anti-inflamatorias o la síntesis de proteínas reparadoras de la matriz, que estabilizan las placas ateroscleróticas vulnerables. Pero también pueden promover efectos deletéreos: además de modular el metabolismo lipídico, los monocitos secretan citoquinas inflamatorias, quimioquinas y especies reactivas de oxígeno que dirigen la patogenia. Asimismo, producen TF y proteasas que contribuyen a la trombosis y la ruptura de la placa aterosclerótica [30].

Diversos estudios en pacientes SAF, LES y RA han demostrado múltiples alteraciones en el estatus de activación y funciones secretoras de monocitos circulantes y tisulares, todas ellas asociadas al desarrollo de trombosis, la alteración de la funcionalidad de las células T y la producción de autoanticuerpos. Adicionalmente, su capacidad fagocítica y actividad como células presentadoras de antígenos se encuentran alteradas en estos pacientes [31]. Por tanto, la modulación farmacológica de la actividad monocito/macrofágica representa una estrategia terapéutica potencial para la prevención de la aterotrombosis.

3.2 Neutrófilos

Los neutrófilos son células integrantes de la respuesta inflamatoria temprana. Tras un estímulo infeccioso o inflamatorio, estas células liberan especies reactivas de oxígenos (EROs) y de nitrógeno (ERNs), se degranulan, secretan serin proteasas y otras enzimas, y producen NETs (redes extracelulares de neutrófilos, del inglés *neutrophil extracelular traps*, constituidas por mallas de cromatina decondensada, histonas, péptidos antimicrobianos y enzimas). Estas estructuras dan origen a diversos estados patológicos que incluyen trombosis (por activación plaquetar e inducción de TF y factor vonWillebrand) [32,33], aterosclerosis (mediante la producción de ROS y la activación leucocitaria) [34] y autoinmunidad (a través del aumento de la carga auto-antigénica) [35].

Numerosas evidencias en la última década han implicado a los neutrófilos en el inicio y perpetuación del LES, así como en el daño orgánico resultante. Los neutrófilos en pacientes LES son activados en el compartimento vascular, sobre-expresan moléculas de adhesión y ROS, y secretan mediadores inflamatorios tales como citoquinas, defensinas,

lactoferrina y otras proteínas bactericidas [21, 36, 37].

En la AR, los neutrófilos son las células más abundantes en el fluido sinovial de articulaciones afectadas y en la interfase pannus-cartílago, donde ocurre el daño tisular. Promueven daño mediante la inducción de estrés oxidativo, liberación de enzimas líticas y expresión de citoquinas proinflamatorias [38].

3.3 Linfocitos

La inmunidad adaptativa afecta igualmente al inicio y la progresión de la aterosclerosis. Se desarrolla cuando antígenos como la LDLox y las proteínas de choque térmico son reconocidas por linfocitos T y B. Aún hay muy pocos datos en EAS en este campo. En pacientes con LES sólo un estudio ha identificado un desequilibrio en la presencia de células Th17/Treg asociado al desarrollo de aterosclerosis [39]. Se ha identificado también un subtipo de células B (CD11bCD20+CD27+CD43+) denominadas células B-1 organizadoras, que están incrementadas en pacientes LES, producen IL10 y suprimen la activación de las células T, con un posible papel ateroprotector [40].

En la AR, los linfocitos son las células más estudiadas como mediadoras de la patogénesis de la enfermedad. Así, la AR se considera un desorden conducido por los linfocitos *helper* o Th, los cuales muestran un desequilibrio en la proporción Th1/Th2 hacia un aumento de los niveles de Th1 en relación a donantes sanos[41]. Los linfocitos Th1 liberan moléculas inflamatorias como interferón- gamma (IFN- γ), interleuquina 2 (IL-2) y factor de necrosis tumoral-alfa (TNF-α), que previenen la diferenciación de las células T $CD4^+$ a Th2. Además, las células Th17 se han asociado

recientemente con la patogénesis de la RA [42], ya que son productoras de IL-17A, IL-6 y TNFα, citoquinas sobreexpresadas en el suero de pacientes AR y responsables de la activación de otras células inmunes y endoteliales.

Las células T reguladoras (Treg) también participan en el curso de esta enfermedad autoinmune. Juegan un importante papel en la inmunosupresión y sobre la interacción célula-célula, y se encuentran significativamente reducidas en estadíos avanzados [41]. Así, se ha propuesto que el desarrollo y progresión de la AR y sus complicaciones clínicas son causadas por un desequilibrio entre las células Th1/Th2 y las Th17/Treg.

En suma, numerosos estudios indican un claro papel de las células integrantes del sistema inmune en la fisiopatología de la aterotrombosis. No obstante, en las EAS es aun necesario un conocimiento detallado de la biología leucocitaria, su interrelación, mecanismos de activación y asociación con el desarrollo de la patología CV.

4. Estrés oxidativo y aterotrombosis en la patología autoinmune

Recientemente se ha demostrado que el estrés oxidativo ejerce un papel fundamental en la patogenia de la aterotrombosis. El estrés oxidativo podría definirse como el desequilibrio bioquímico propiciado por la producción excesiva de especies reactivas y radicales libres que genera daño oxidativo en las biomoléculas y que no puede ser contrarrestado por los sistemas antioxidantes.

En condiciones fisiológicas normales, las especies reactivas de oxígeno y nitrógeno son producidas por la mitocondria como resultado del

metabolismo aerobio, los peroxisomas celulares en procesos de detoxificación, o la NADPH oxidasa de las células fagocíticas, como sistemas de señalización celular y como medio del sistema inmunitario para eliminar patógenos. Estas especies reactivas y radicales libres (peróxido de hidrógeno, óxido nítrico, anión superóxido, peroxinitritos, radical hidroxilo y alcoxilo, etc.) son luego eliminados o neutralizados por las defensas antioxidantes del organismo, tales como las enzimas catalasa (CAT), superóxido dismutasa (SOD), glutatión peroxidasa (GPx), y algunas vitaminas. Sin embargo, cuando este equilibrio se pierde, por procesos inflamatorios o infecciosos, por ejemplo, se incrementa la concentración de especies reactivas de oxígeno (EROs) y nitrógeno (ERNs), conduciendo a un daño en los componentes celulares, tales como los lípidos o las proteínas, alterando el metabolismo y crecimiento celular normal y dando lugar a alteraciones en el sistema de defensa, el envejecimiento o numerosas enfermedades (Figura 3).

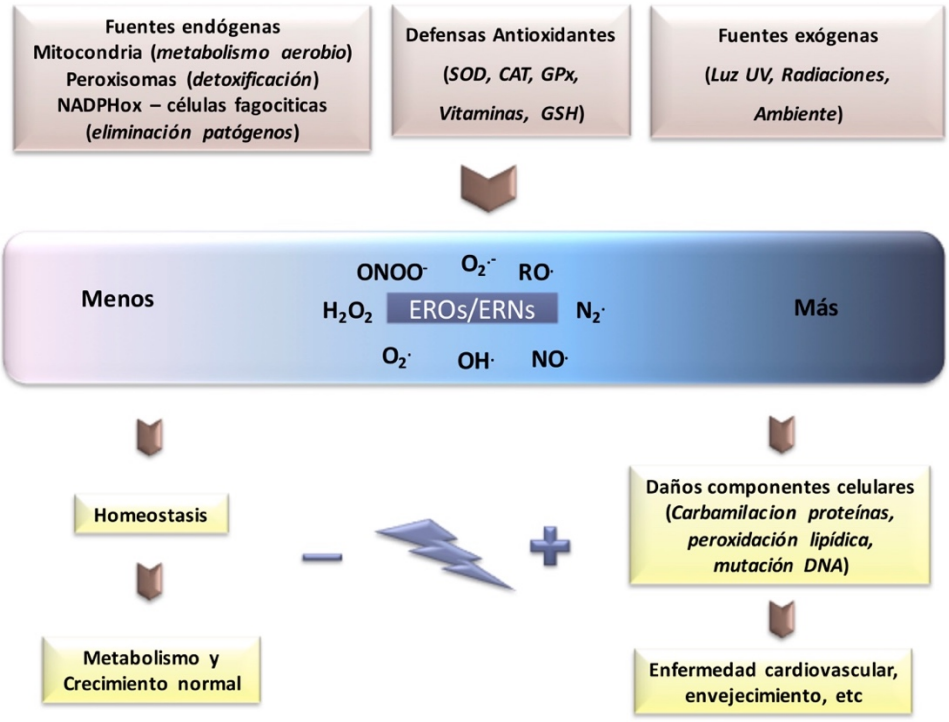

Figura 3. Fuentes y respuesta celular a Especies Reactivas de Oxígeno (EROs) y nitrógeno (ERNs). Abreviaturas: NADPHox, nicotinamida adenina dinucleótido fosfato-oxidasa; SOD, superóxido dismutasa; CAT, catalasa; GPx, glutatión peroxidasa; GSH, glutatión reducido.

El estrés oxidativo, a su vez, participa en el estado inflamatorio, ya que una excesiva producción de EROs/ERNs puede modular la expresión de quimioquinas inflamatorias [43, 44]. El incremento en los niveles de quimioquinas en el suero y el desequilibrio en el estrés oxidativo promueven a su vez el reclutamiento de leucocitos a órganos y tejidos, facilitando así la inflamación sistémica que sufren los pacientes autoinmunes y que favorece el desarrollo de la ECV.

4.1 Estrés oxidativo en pacientes con Síndrome Antifosfolípido

En patologías autoinmunes como el SAF o el SAF+LES se ha demostrado que los anticuerpos aCL juegan un papel relevante en la inducción de un estatus prooxidativo, a través de la inducción de la producción de óxido nítrico y anión superóxido, con la consecuente generación de peroxinitritos, potentes sustancias prooxidantes asociadas con disfunción vascular y aterogénesis [45]. Los títulos de aCL también se han correlacionado con los niveles plasmáticos de F2-isoprostanos, marcadores de peroxidación lipídica *in vivo*. Otro mecanismo pro-aterogénico promovido por los AAF es la inducción del consumo de LDLox por parte de los macrófagos. Este efecto concuerda con la reducción de la actividad paraoxonasa encontrada en estos pacientes. La paraoxonasa es una enzima antioxidante asociada HDL que previenen la oxidación de las LDL. Es más, la actividad de esta enzima se ha asociado a la presencia de alteraciones estructurales y funcionales del sistema arterial en pacientes SAF. A nivel molecular estos autoanticuerpos promueven la activación constitutiva del NFkB, y consecuentemente la activación de la enzima óxido nítrico sintasa, ambas responsables de la disfunción endotelial característica de estos pacientes.

En este ámbito, un estudio reciente ha descrito la presencia de una disfunción mitocondrial en los monocitos de pacientes SAF, mediada por los AAFs. Dichos autoanticuerpos promueven una fisión mitocondrial que se traduce en el incremento en los niveles de EROs, los cuales actúan como segundos mensajeros induciendo la expresión de proteínas protrombóticas y proinflamatorias en los monocitos de estos pacientes, favoreciendo así un estado proaterotrombótico [17].

4.2 Estrés oxidativo en pacientes con Lupus Eritematoso Sistémico

En el entorno del LES, el estrés oxidativo juega un papel muy relevante en la patogénesis de la enfermedad, a través de tres mecanismos principales: potenciación de la inflamación, inducción de apoptosis y ruptura de la tolerancia inmunológica. La generación de EROs/ERNs proporciona moléculas inductoras de un daño en la membrana lipídica de la célula, la oxidación proteica y el daño en el ADN. La reducción en la eliminación de cuerpos apoptóticos (por un defecto en la actividad fagocítica de los macrófagos presente en estos pacientes) facilita la interacción de moléculas intracelulares con las EROs, lo que sienta las bases para la generación de nuevos autoanticuerpos en el LES. La depleción de glutatión, característica de estos pacientes, favorece esta cascada, puesto que puede conducir a un aumento transitorio del glutatión en su forma oxidada (GSSG), precipitando la apoptosis por activación de la enzima caspasa 3, y promoviendo como consecuencia una mayor severidad de la enfermedad [46].

Otros estudios han confirmado asimismo que los linfocitos T muestran hiperpolarización mitocondrial, expresión incrementada de EROs, niveles reducidos de glutatión intracelular (GSH), alcalinización citoplásmica, y depleción de ATP, lo cual sensibiliza a los linfocitos T a la necrosis [47].

4.3 Estrés oxidativo en pacientes con Artritis Reumatoide

En la AR el estrés oxidativo juega un importante papel en el desarrollo de la enfermedad. Numerosos estudios han demostrado una alteración de los niveles de EROs plasmáticas en pacientes AR en relación a donantes sanos, aunque los estudios a nivel celular son escasos [48]. Los radicales

libres están indirectamente implicados en el daño tisular ya que juegan un importante papel como segundos mensajeros en la respuesta celular en AR. Así, se ha demostrado que linfocitos T expuestos a un incremento de estrés oxidativo se hacen refractarios a diferentes estímulos, incluyendo aquellos asociados al crecimiento y muerte, perpetuando así la respuesta inmune alterada [49].

Los niveles de EROs se han visto asimismo correlacionados con la actividad de la enfermedad (DAS28). Es más, estos radicales libres dañan el cartílago articular, afectando a los proteoglicanos e inhibiendo su síntesis [50].

También se ha demostrado la existencia de un daño oxidativo sobre el ácido hialurónico, proteínas y ADN, así como una oxidación elevada de las LDLs que podrían contribuir al desarrollo de la AR y de la ECV asociada [48]. Junto a todo ello, también se ha observado que en la AR existe un sistema antioxidante deficitario, con bajos niveles de glutatión reducido [51], tocoferoles, β-carotenos y retinoles y bajas actividades de las enzimas superóxido dismutasa (SOD) y glutatión reductasa (GR) [52].

No obstante, aún no se ha analizado el estado oxidativo presente en los distintos subtipos de células inmunes en pacientes con EAS, su asociación con el estado autoinmune e inflamatorio, y su papel en el desarrollo de aterotrombosis.

5. Mecanismos genéticos y epigenéticos involucrados en la regulación de la aterotrombosis en enfermedades autoinmunes sistémicas.

El conocimiento de los mecanismos fisiopatológicos involucrados en el desarrollo de aterosclerosis y enfermedad cardiovascular en las EAS se ha ampliado de forma considerable con la aplicación de novedosas tecnologías genómicas (microarrays y secuenciación masiva para la

detección de alteraciones en la expresión génica, *splicing* alternativo, polimorfismos etc), las cuales permiten explicar cómo estas alteraciones podrían estar asociadas a cada enfermedad autoinmune a nivel molecular [53].

Numerosas evidencias apoyan la existencia de un componente hereditario en el desarrollo de las patologías autoinmunes. Datos epidemiológicos sugieren que concurre una alteración genética subyacente que contribuye a la susceptibilidad de la enfermedad [54–56]. Así, se han identificado regiones génicas donde reside una alta susceptibilidad a padecer enfermedades autoinmunes, destacando el locus HLA. En este sentido, desde hace años se ha descrito que diversos alelos HLA-DR o HLA-DQ se asocian con susceptibilidad a enfermedades autoinmunes como la AR, la diabetes mellitus tipo 1 y el LES.

Se ha descrito también la existencia de zonas génicas susceptibles que son comunes para la mayoría de enfermedades autoinmunes y otras que son específicas del tipo de enfermedad autoinmune analizado [57, 58].

Varios trabajos han comparado el perfil de expresión génica en células mononucleadas de sangre periférica de pacientes EAS e individuos sanos, habiéndose demostrado un patrón de expresión definido que identificaba genes distintivos en dichas patologías [59-63]. Sin embargo el estudio del perfil génico en distintos tipos celulares del sistema inmune, no realizado hasta el momento, podría proporcionar información más precisa acerca de genes específicos diferencialmente expresados, lo cual supondría una ventaja en la búsqueda de nuevos biomarcadores para el diagnóstico y pronóstico de distintas EAS, así como de sus comorbilidades asociadas.

Aunque la mayoría de las células posee la misma secuencia de ADN, la actividad de los genes individuales difiere significativamente entre diferentes tipos celulares y tejidos, dependiendo no sólo de la secuencia de nucleótidos del ADN, sino también de una gran diversidad de mecanismos reguladores. La epigenética involucra todos los mecanismos no genéticos (no explicables debido a la secuencia del ADN) que dan lugar a una expresión genética diferencial, es decir, alteran la expresión génica y proteica y, por tanto, definen el fenotipo del organismo. Se podría definir como el estudio de los cambios heredables en la estructura y organización del ADN que no involucran cambios en la secuencia y que modulan la expresión génica, e implican cambios heredables en el fenotipo [64, 65].

La epigenética comprende la metilación del ADN, modificación de histonas y la actividad de los microRNAs (miRNAs), proporcionando nuevos vínculos entre la genética y los factores ambientales [66].

5.1 Metilación del ADN

La metilación del ADN consiste en la adición de un grupo metilo a la posición 5' del anillo pirimidínico de las citosinas que van seguidas de guaninas, denominados nucleótidos CpG. Estos dinucleótidos CpG tienden a concentrarse en la región promotora de aproximadamente un 60% de los genes codificantes en humanos, configurando las Islas CpG (regiones entre 200-2000 bases con una proporción de CG superior al 50%). Estas islas se encuentran comúnmente metiladas en genes silenciados e hipometiladas en genes transcripcionalmente activos, permitiendo la actividad génica cuando los factores de transcripción correspondientes están disponibles. Las enzimas encargadas de metilar el ADN son las DNMTs o ADN metiltransferasas [67].

5.2 Modificación de histonas

Las histonas (H2A, H2B, H3 and H4), lejos de desempeñar una función meramente estructural como proteínas empaquetadoras del ADN, sufren cambios postraduccionales que tienen repercusión directa sobre la conformación de la cromatina, participando en procesos de reparación del ADN, actividad transcripcional, y replicación, entre otros. Las modificaciones más estudiadas que se dan en histonas son la acetilación, metilación de lisinas y argininas, y fosforilación de serinas. La acetilación de histonas ocurre por acción de enzimas acetil transferasas (HATs), mientras que su desacetilación resulta de la actividad de enzimas desacetilasas (HDACs). En general, la acetilación de lisinas se correlaciona con activación transcripcional, debido a su efecto en las interacciones electrostáticas entre histonas y ADN, mientras que el proceso contrario se asocia con silenciamiento. La metilación de histonas afecta a lisinas y está asociada tanto a activación como a represión transcripcional [68].

5.3 microRNAs

Los microRNAs son pequeños ARNs monocatenarios, no codificantes, de aproximadamente entre 19 y 25 nucleótidos, que se originan en el núcleo como pri-miRNAs tras ser transcritos por la RNA polymerase II/III. Posteriormente son procesados por la ribonucleasa Drosha y se transportan al citosol a través de la proteína exportina-5 como pre-miRNAs. Una vez en el citosol una nueva ribonucleasa, Dicer, lo vuelve a escindir generando dos moléculas complementarias, para que finalmente una de ellas se ensamble en el complejo RISC (RNA-*induced silencing complex*) donde el miRNA maduro es funcional y activo[69] (Figura 4).

Su mecanismo de acción consiste en la regulación negativa de la expresión génica a nivel post-transcripcional. Así, cuando el miRNA maduro encuentra una complementariedad exacta con una secuencia en el extremo 3´UTR del ARN mensajero (ARNm) de su gen diana, lo degrada. Si la complementariedad que encuentra el miRNA con su ARNm diana es parcial, la traducción a proteína es reprimida [69, 70].

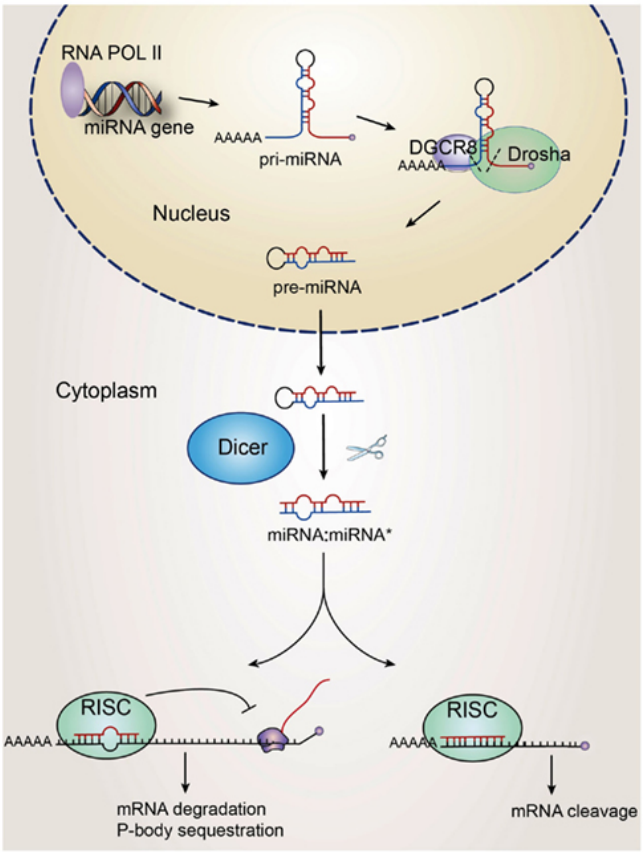

Figura 4. Biogénesis de miRNAs y mecanismo de acción. (Barca-Mayo O and Lu QR. Front Neurosci. 2012). Los miRNAs se transcriben en el núcleo gracias a la RNA pol II/III. Su conformación inmadura (pri- y pre-miRNAs) comprende el procesamiento mediado por diferentes ribonucleasas como Drosha y Dicer. Finalmente el miRNA maduro se asocia a otras proteínas como Argonauta y TRBP en el Complejo RISC donde lleva a cabo la regulación post-transcripcional de sus ARNm dianas.

Hasta la fecha, la base de datos miRBase ha catalogado más de 35 mil miRNAs maduros en 223 especies, entre los que 2588 corresponden a secuencias humanas de miRNAs maduros. Estos pueden regular hasta el 60% de los genes humanos [71].

La metilación del ADN, la modificación de histonas y la actividad de miRNAs constituyen procesos epigenéticos que funcionan de forma coordinada para regular de forma precisa la expresión del genoma, habiéndose demostrado su papel clave en numerosos procesos fisiológicos y patológicos (carcinogénesis, diferenciación hematopoyética, inmunidad innata y adquirida, oncogénesis, autoinmunidad, etc.).

En los apartados siguientes nos centraremos en el estudio de los miRNAs como ejemplo de mecanismo epigenético con un papel clave en el desarrollo de enfermedades autoinmunes sistémicas.

5.3.1 miRNAs asociados a Enfermedades Autoinmunes Sistémicas

Diversos trabajos han analizado el perfil de expresión de miRNAs en células de sangre periférica, fluidos biológicos y tejidos de pacientes con EAS [72, 73]. En LES, se han identificado múltiples miRNAs diferencialmente expresados, que parecen contribuir a la patogénesis de la enfermedad por medio de la regulación de la ruta del interferón tipo I, la expresión de citoquinas inflamatorias, la metilación del ADN en células T y la inflamación local (i.e. miR-15, miR-21, miR-31, miR-125a, miR142, miR-146a, miR-155, and miR-181, entre otros) [74]. Además, la expresión alterada de miRNAs en pacientes LES, influencia algunos parámetros asociados con la severidad y actividad de la enfermedad [71, 75].

En el ámbito del SAF, un estudio reciente ha demostrado que diversos miembros del *cluster* miR 17-92 (miR-20a y miR-19b), inicialmente identificados como reguladores de la angiogénesis tumoral, también regulan la expresión de TF en monocitos, de modo que se ha observado una reducción en su expresión directamente asociada a la sobre activación del TF [76]. La reducción en la expresión de estos miRNAs podría así contribuir al estado protrombótico característico de pacientes SAF. Se trata del primer trabajo que muestra que los miRNAs pueden ser importantes moduladores del sistema hemostático y estar implicados en procesos fisiopatológicos asociados a la patología cardiovascular en esta enfermedad autoinmune.

En AR, múltiples estudios han mostrado una alteración en la expresión de miRNAs en células mononucleares de sangre periférica [77] y linfocitos T [78], así como en tejido y fibroblastos sinoviales [79-81], con un papel clave en procesos como inflamación, degradación de matriz extracelular y comportamiento invasivo de células residentes. También se ha demostrado un perfil de expresión alterada en plasma y fluido sinovial de pacientes con AR [85], incluso en diferentes estados de progresión de la enfermedad, lo que ha llevado al reconocimiento de los miRNAs como biomarcadores de la actividad de la enfermedad e incluso de la respuesta terapéutica [83, 84].

5.3.2 miRNAs asociados a enfermedad cardiovascular

Son numerosos los estudios que han identificado el papel de los miRNAs en procesos como el estrés oxidativo, la homeostasis y la enfermedad cardiovascular [85-87] (Figura 5).

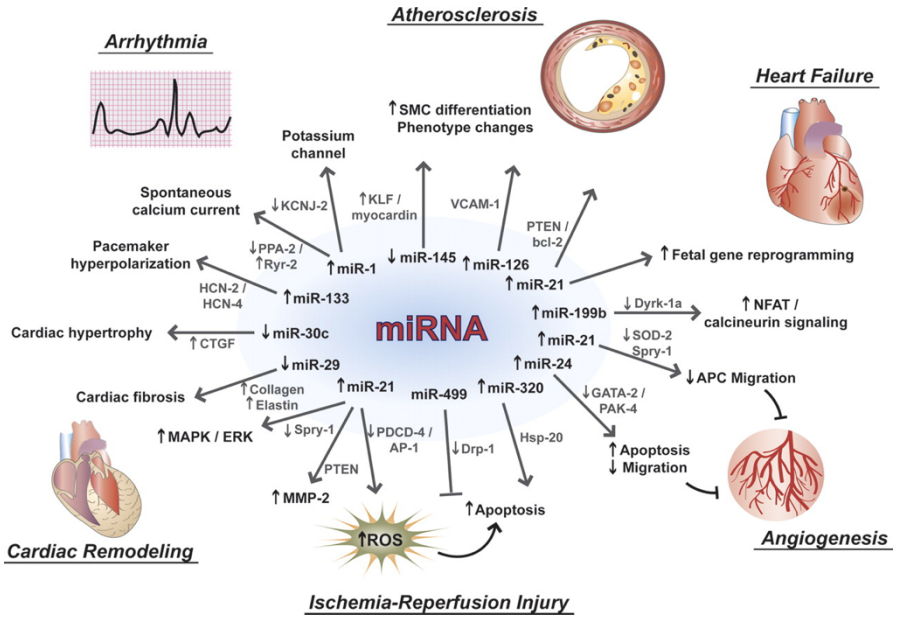

Figura 5. miRNAs y dianas asociadas a patologías cardiovasculares. (S. Danwal *et al*. Cardiovascular Research. 2013). Diferentes miRNAs han sido descritos como reguladores de proteínas claves mediadoras de procesos patológicos cardiovasculares, cuya expresión se ha encontrado alterada.

Estos trabajos han descrito alteraciones en los niveles de expresión de miRNAs que controlan dianas moleculares importantes en el contexto oxidativo, cardiovascular y hemostático (V-CAM, SOD-2, Colágeno, KLF, Factor XI, TFPI etc.), tanto a nivel plasmático como celular en patologías tales como el infarto de miocardio, la aterosclerosis, la enfermedad arterial coronaria, el fallo cardíaco, la hipertrofia, la fibrosis y la fibrilación auricular. Ello ha propiciado que varios de ellos sean considerados biomarcadores de enfermedad cardiovascular (i.e. miR-21, miR-145, miR-126, miR-199, miR-29 etc.) [86, 87]. Sin embargo, ningún estudio ha identificado y caracterizado los miRNAs asociados a la enfermedad cardiovascular y al riesgo aterotrombótico que presentan los pacientes con EAS.

En suma, en base a los antecedentes descritos, el conocimiento profundo de la fisiopatología de enfermedad cardiovascular en las EAS requiere un

análisis integrado de los mecanismos autoinmunes, moleculares, genéticos y epigenéticos que controlan dicha comorbilidad. Ello conduciría a un conocimiento más profundo de su origen y modulación, propiciando el desarrollo de estrategias terapéuticas futuras más eficientes y personalizadas.

6. Referencias

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HIPÓTESIS

Hipótesis

El desarrollo de aterosclerosis y enfermedad cardiovascular en pacientes con patologías autoinmunes sistémicas como el Síndrome Antifosfolípido, el Lupus Eritematoso Sistémico y la Artritis Reumatoide, podría estar relacionado con la existencia de una predisposición intrínseca localizada a dos niveles distintos, aunque interrelacionados entre sí: la regulación de las respuestas inmunes innata/adaptativa y el sistema coagulativo, que conforman mecanismos biológicos altamente conservados.

Se ha descrito la implicación de los auto-anticuerpos en el desarrollo de aterotrombosis en SAF, LES y AR. Sin embargo, aún es necesario analizar en detalle qué auto-anticuerpos y a qué nivel contribuyen al desarrollo de la ECV en cada condición autoinmune, así como los mecanismos moleculares subyacentes.

Las células integrantes del sistema inmune constituyen igualmente piezas clave en el desarrollo de la patología CV en el entorno de las EAS. Por tanto, es crítica la caracterización del papel de los subtipos leucocitarios en el desarrollo de aterotrombosis en las diferentes EAS, así como su interacción y mecanismos de activación.

El estrés oxidativo juega asimismo un papel determinante en el desarrollo de aterosclerosis. La alteración de la biogénesis y la función mitocondrial son igualmente procesos moleculares ligados a la fisiopatología CV de las EAS. Por tanto, el análisis de las causas del fallo en el sistema de detoxificación que resulta en una producción mitocondrial excesiva de EROs/ERNs presentes en los distintos subtipos de células inmunes y su asociación con el estado inflamatorio, es un paso esencial en el

conocimiento de la fisiopatología de la aterotrombosis de origen autoinmune.

Estudios recientes indican que los miRNAs desempeñan un papel muy relevante y novedoso en la regulación de la expresión de genes y proteínas clave en la patología aterotrombótica. Por tanto, la identificación y caracterización de los miRNAs implicados en el control de la expresión de proteínas proinflamatorias y protrombóticas en EAS podría contribuir a la tipificación de nuevos biomarcadores de patología cardiovascular.

En suma, el desarrollo de aproximaciones metodológicas que integren el análisis de elementos inmunológicos, celulares, moleculares y epigenéticos permitiría identificar nuevos mecanismos reguladores de la patología cardiovascular en enfermedades autoinmunes sistémicas, estableciendo biomarcadores de enfermedad susceptibles de ser modulados utilizando nuevas aproximaciones terapéuticas.

OBJETIVOS

Objetivos

En enfermedades autoinmunes sistémicas (EAS) como la Artritis Reumatoide (AR), el Lupus Eritematoso Sistémico (LES) o el Síndrome Antifosfolípido (SAF), se presentan eventos coronarios agudos sin que medien factores de riesgo tradicionales previos que los justifiquen. En estas patologías autoinmunes el riesgo aterotrombótico es muy elevado, ya que los procesos inflamatorios crónicos que las caracterizan incrementan la probabilidad de eventos trombóticos y contribuyen al desarrollo prematuro de placas ateroscleróticas, o aceleran la desestabilización de aquellas ya formadas. Sin embargo, las características moleculares específicas que definen cada enfermedad pueden asimismo contribuir a determinar mecanismos únicos de patología cardiovascular (CV) en cada condición autoinmune.

Por lo tanto, el objetivo principal de esta Tesis doctoral ha sido el estudio de los mecanismos subyacentes al desarrollo de la aterotrombosis en enfermedades autoinmunes sistémicas (SAF, LES y AR), mediante la integración de análisis inmunológicos, celulares, moleculares y epigeneticos.

Objetivos específicos:

1- Definir el papel específico de los anticuerpos anti antipéptidos cíclicos citrulinados (anti-CCPs) sobre el perfil pro-oxidativo, inflamatorio y proaterogénico observado en leucocitos de pacientes con AR.

> Artículo: *Anti-cyclic citrullinated protein antibodies are implicated in the development of cardiovascular disease in rheumatoid arthritis. Arteriosclerosis, Thrombosis, and Vascular Biology (ATVB). 2014*

2- Identificar genes y rutas moleculares comunes y divergentes involucradas en la patogénesis de la aterosclerosis y enfermedad cardiovascular en el Lupus Eritematoso Sistémico (LES), el Síndrome Antifosfolípido Primario (SAF) y el Síndrome Antifosfolípido asociado a Lupus (SAF+LES).

Artículo: *Gene profiling reveals specific molecular pathways in the pathogenesis of atherosclerosis and cardiovascular disease in Antiphospholipid syndrome, Systemic Lupus Erythematosus and Antiphospholipid Syndrome with Lupus. Annals of the Rheumatic Diseases* (*ARD*). *2015*

3- Identificar y caracterizar los miRNAs implicados en el desarrollo de ECV en SAF y LES y evaluar, in vitro, el efecto de auto anticuerpos específicos de SAF (aPL) y LES (anti-dsDNA) sobre su regulación.

Artículo: *Atherothrombosis-associated microRNAs in Antiphospholipid syndrome and Systemic Lupus Erythematosus patients.* Scientific Reports *(*Sci. Rep.*). 2016*

CAPÍTULO I

Arteriosclerosis, Thrombosis, and Vascular Biology
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Anticyclic Citrullinated Protein Antibodies Are Implicated in the Development of Cardiovascular Disease in Rheumatoid Arthritis

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1. ABSTRACT

Objective: Previous studies have suggested a relationship between anti-CCPs levels and development of cardiovascular disease in rheumatoid arthritis (RA). However, a limited number of studies have demonstrated an involvement of anti-CCPs in those processes. This study was aimed to define the specific role of these auto-antibodies in the pro-oxidative, inflammatory and pro-atherogenic profile observed in leukocytes from RA patients.

Approach and results: 75 RA patients and 31 healthy donors were enrolled. Carotid intima-media thickness (CIMT) was evaluated as atherosclerosis marker. Several pro-coagulant and inflammatory factors, leukocyte activation, and oxidative stress markers, were analyzed in plasma and leukocyte subsets. Anti-CCPs were purified from plasma of RA patients, and in vitro treatment of healthy leukocytes was conducted. High titers of anti-CCPs were associated to altered expression of pro-thrombotic and inflammatory markers, high oxidative stress and pathological CIMT in RA patients. Notably, gene expression analysis showed that lymphocytes were mayor players in altered inflammatory profile, monocytes were responsible for the protrombotic and atherogenic status, and neutrophils mainly displayed a pro-oxidative feature. In vitro treatment with purified anti-CCPs fully recapitulated that pathogenic profile, promoting the activation of leukocytes.

Conclusions: Anti-CCPs are key players in the inflammatory and pro-atherogenic status of RA patients. The effects are specific of the immune cell targeted, promoting over-expression of thrombotic, inflammatory and pro-oxidative markers in monocytes, pro-oxidative status in neutrophils, and pro-inflammatory profile in lymphocytes. Targeting these

autoantibodies would be an excellent strategy to prevent the development of cardiovascular disease in RA.

2. INTRODUCTION

Rheumatoid arthritis (RA) is a disorder comprising a complex onset mechanism and many associated complications. In particular, cardiovascular disease (CVD) significantly contributes to morbidity and mortality in these patients, causing the 39-50 % of deaths (1) and atherosclerosis at very early stage of the disease is considered as a possible preclinical manifestation. In fact, the risk of CVD events such as myocardial infarction is increased in the 2 years preceding formal diagnosis of RA (2) and once the disease is diagnosed the risks of carotid plaques and CVD events increase with the duration of RA (3). Thus, the strong relationship between atherosclerosis and RA has led some authors to include atherosclerosis among the extra-articular manifestations of the disease (4).

The mechanisms responsible for the premature atherosclerosis in RA are not well understood, but traditional risk factors alone are not fully responsible and a role of inflammation in this process has been suggested. Considering that RA is a chronic inflammatory disease and the pathogenic role of inflammation in the atherosclerosis (5) it is likely that inflammatory mediators might be causal in the accelerated atherosclerosis observed in RA.

Oxidative stress is another process frequently altered in RA that also contributes to atherosclerosis, being associated with a poorer prognosis. Neutrophils are thought to be responsible for the elevated levels of reactive oxygen species (ROS) found in plasma and synovial fluid of RA

patients, thus contributing to the joint tissue damage within synovial membrane (6). In addition, a positive correlation has recently been shown between neutrophils ROS levels in blood and synovial fluid with DAS28, C reactive protein (CRP) and anti-CCPs levels (7). However, monocytes also seem to contribute to the elevated oxidative stress in blood of RA patients through the production of superoxide anion (8).

Many different cell components can be considered as key elements in the long course of RA, including blood white cells such as lymphocytes, monocytes and neutrophils. Activation of these cells leads to the production of cytokines and mediators of inflammation, oxidative stress and atherosclerosis. In this regard, monocytes/macrophages are central players in inflammation and have been found to be activated in RA, through the release of cytokines (9) and massively infiltration in inflammatory sites such as synovial membranes (10). In addition, monocytes/macrophages have been associated with bone erosion in RA, owing to their excessive differentiation into osteoclasts (11). Due to its role in RA, several monocyte-targeted therapies are being developed.

Lymphocytes are the most studied cells mediating the pathogenesis of RA, which in fact is considered a T helper 1 (Th1)-cell-driven disorder with a Th1/Th2 imbalance towards higher Th1 levels compared to healthy donors (12). Th1 releases inflammatory molecules such as IFN-γ, IL-2 and TNF-α that prevent CD4+T cells from differentiating into Th2 cells. Moreover, Th17 has lately been associated with RA pathogenesis (13), mainly producing IL-17A, IL-6 and TNF-α, cytokines that are increased in serum of RA patients, and are responsible for the activation of other cells. On the other hand, regulatory T cells (Treg) also participate in the course of this

disease. They play an important role in immunosuppression through cell-cell interaction, are found significantly decreased at advanced stage and their function has been shown impaired in RA (12). Thus, it has been proposed that the development and progression of RA is caused by the imbalance of Th1/Th2 and Th17/Treg cells.

Neutrophils are the most abundant cells in the synovial fluid of affected joints and at the pannus/cartilage interface, where tissue damage occurs. They promote damage through the induction of oxidative stress, enzyme release and expression of proinflammatory cytokines. However, the role of neutrophils in RA pathogenesis is not fully understood (14).

Among the auto-antibodies detected in RA, the anti-cyclic citrullinated peptides (anti-CCPs) are highly specific for this disease (15), since IgM rheumatoid factor (RF) is also present in an elevated percentage of patients with a number of chronic infections. Moreover, atherosclerosis status in RA is independent of RF levels (16). In contrast, detection of anti-CCP antibodies has become a useful diagnostic tool, particularly at very early stage (17). Thus, the new 2010 RA classification criteria included detection of anti-CCPs as a key item for diagnosing the disease (18).

High levels of anti-CCPs have been suggested to predict an adverse cardiovascular profile in RA patients. However, that hypothesis just arises from association studies in various cohorts, and no study has still demonstrated a direct effect of anti-CCPs in all those processes.

Here we demonstrate the direct involvement of anti-CCPs, purified from RA patients, in the induction of a pro-oxidative, pro-inflammatory and atherogenic status in different leukocytes subsets. In addition, specific effects on macrophage activation and survival were also demonstrated.

3. MATERIAL AND METHODS

3.1 Patients

Seventy-five RA patients and 31 age and gender-matched healthy donors were included in this study. RA patients fulfilled at least four 1987 American College of Rheumatology (ACR) and achieved a total score of 6 or greater according to 2010 criteria (18, 19). The patients were given the following treatments: Corticosteroids (54.6 %), antimalarials (16%), NSAIDs (78.6%), Methotrexate (68%) and Vitamin D (36%). All patients were tested for the presence of anti-CCPs and RF. Disease activity score 28 (DAS28) index was determined following the guidelines of the American college of Rheumatology indications (DAS28 3.49 ± 0.17); moderate to high activity was defined as DAS28 ≥3.2 (20). None of the healthy donors had a history of other autoimmune diseases, atherothrombosis and thrombosis.

For in vitro studies, serum was further obtained from 4 ankylosing spondylitis patients (AS) meeting the modified New York criteria for AS (21). They all were male, between 46 and 70 years old, CRP range from 10.4 to 60 mg/ml and ESR range from 9 to 30.

All participants enrolled were Caucasian and recruited at the department of Rheumatology, and gave their written informed consent approved by the ethical committee of the Reina Sofia Hospital (Cordoba, Spain).

3.2 B-Mode Ultrasound IMT Measurements

RA patients and controls underwent B-mode ultrasound imaging for CIMT (carotid intima media thickness) measurements. B-mode ultrasound imaging of the carotid arteries was performed by using Toshiba

equipment (Aplio platform) equipped with 7–10 MHz broadband linear array transducers.

Plaque was defined as a focal structure that encroached into the arterial lumen of at least 50% of the surrounding IMT value or demonstrated a thickness >1.5 mm, as measured from the media-adventitia interface to the intima-lumen interface.

3.3 Inflammatory markers in plasma: Flowcytomix

VEGFa, sCD40L, IFNγ IL-2, IL-6, IL-8, IL-10, IL-17A, IL-23, MCP-1, MIP-1α, MMP-13, sP-selectin, TNF-α and tPA were quantified using a cytofluorimetry-based ELISA system following the manufacturer's recommendations (Flowcytomix, Bender Medsystem GmbH, Austria). Two-colour cytometry analysis was performed using FACScalibur cytometer (BD Biosciences, San Jose, California, USA). Data were obtained and analysed using the FlowCytomix Pro software.

3.4 Expression of CD14 by flow cytometry

Flow cytometric analysis was performed using a FACScalibur (BD Biosciences) and a specific monoclonal antibody to human CD14 PE-conjugated (Caltag, South San Francisco, California, USA). Non-specific antibody conjugated to PE was used as negative control.

3.5 White blood cells isolation

Neutrophils were isolated from patients and healthy donors blood by density centrifugation over Dextran-Ficoll Hypaque as described by Nauseef NM (22), which allows cells to keep a non activated state. Thereafter, the separation of monocytes and lymphocytes from the

mononuclear layer was performed by the immunomagnetic depletion of non-monocytes, using a commercially available kit (Monocyte isolation kit II, Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the fractions was evaluated via flow cytometry (FACScalibur cytometer) analyzing the size and complexity of each population (forward and side scatters). The purity of monocytes was further evaluated with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 antibody and flow cytometry. By this method, 93.5 ± 2.5 viable monocytic cells were obtained.

3.6 Purification of anti-CCPs from RA patients and in-vitro culture of white blood cells

IgG-CCPs antibodies from the pooled sera of 7 RA patients (characterised by high titres of anti-CCPs >100 U/ml) were purified using a commercial kit (Euro-Diagnostica, Nijmegen, The Netherlands). 100 μl of pooled sera were added to the microtitres plates coated with citrullinated synthetic peptides which allowed the binding of the antibodies (anti-CCPs). Next, anti-CCPs were eluted with 100 μl/well 0.1 M glycine/0.3 M NaCl pH 2.8 for 20 minutes at room temperature, then neutralised with 2M Tris-HCl pH 7.6 solution. Thereafter, the eluates were concentrated using Amicon Ultra-0.5 centrifugal filter devices from Merk Millipore (Darmstadt, Germany) following the manufacturer's recommendations.

To assess the anti-CCPs activity of these samples, 1:100 diluted samples were tested using the same commercial kit utilized for anti-CCPs isolation (Euro-Diagnostica) according to the manufacturer's instructions.

For in-vitro studies: monocytes, neutrophils and lymphocytes purified from healthy donors were cultured separately in RPMI 1640 containing 10% FBS, 2 mM l- glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin

and 250 pg/ml fungizone (BioWhittaker/MA Bioproducts, Walkersville, Maryland, USA), for 1 or 6 hours at 37°C in a humidified 5% carbon dioxide atmosphere. After 6 hours of culture, the cell death rate of the different cell subtypes was 4.88 ± 2.44% in lymphocytes, 9.39 ± 4.86% in monocytes and 5.35 ± 1.55% in neutrophils. Monocytes, neutrophils and lymphocytes (1×10^6 cells/ml) were incubated with synthetic human IgG (equal protein concentration that the anti-CCPs treatments) (Jackson InmunoResearch Laboratories, Inc, Newmarket, Suffolk, UK) or purified anti-CCPs from the pooled sera of 7 RA patients (5 U/ml).

3.7 Affinity depletion of anti-CCPs from RA patients and in-vitro culture of white blood cells.

IgGs from serum of different RA patients with high titers of anti-CCPs and negative for rheumatoid factor, pooled sera from 4 healthy donors and pooled sera from 4 AS patients were isolated using HiTrap protein G HP columns (GE Healthcare). Anti-CCPs from IgGs-RA patients were purified using Streptavidin agarose columns (Thermo Fisher Scientific, Waltham, MA, USA) coupled with biotinylated CCP peptides following the manufacturer's recommendations (EZ-Link Sulfo-NHS-LC-LC-Biotin, Thermo Fisher Scientific). A cyclic-CCP peptide was synthesized according to the sequence previously designed by Schellekens et al (23) for the clinical test of anti-CCPs (Immunostep, Salamanca, Spain): HQCHQESTXGRSRGRCGRSGS, where X refers to citrulline. The IgGs-containing anti-CCPs from RA serum were incubated with the CCP affinity column 1 hour at room temperature. After incubating the sample, the column was washed with PBS to obtain the flow through with IgGs

depleted in anti-CCPs. Thereafter, antibodies were eluted with 0.1M Glycine-hydrogen chloride (HCL) pH2.5 and neutralised with 2M Tris.

Anti-CCP-IgG activity of starting material (purified IgGs) and flow-through (IgGs depleted in anti-CCPs) was measured by ELISA (Eurodiagnostica). To confirm the specificity of anti-CCPs, monocytes, lymphocytes and neutrophils ($1.5x10^6$ cells) from two healthy donors were treated with: a) 500 μg IgGs from 3 RA patients depleted or non-depleted in anti-CCPs, b) 500 μg IgGs from patients with another inflammatory non autoimmune disease (ankylosing spondylitis) and similar ESR rate to the RA samples used, c) 500 μg IgGs from healthy donors and d) human monoclonal anti-CCP (anti-citrullinated fibrinogen immunoglobulin, clone 1F11, 10 μg/ml) (MQR 2.101-100, Modiquest, Molenweg, The Netherlands) for 6 hours at 37ºC.

Prior to the different treatments, cells were treated with human anti-FCR (Miltenyi Biotec, Madrid, Spain) to block non-specific binding for 30 minutes.

3.8 Characterization of anti-CCPs IgGs subtypes

Anti-CCPs-IgGs isolated from 15 RA patients were characterized using a human IgG subclass ELISA (Life technologies, Madrid, Spain) following the manufacturer's instructions.

3.9 RNA isolation and quantitative real-time reverse transcriptase PCR

Total RNA from lymphocytes, monocytes, neutrophils and THP-1-derived macrophages was extracted using TRI Reagent (Sigma, St Louis, Missouri, USA) following the manufacturer's recommendations. The integrity of

RNA was verified by optical density (OD) absorption ratio OD260/OD280 between 1.8 and 2.0.

For first strand cDNA synthesis, 1µg of total RNA was reverse transcribed using random hexamers as primers and Transcriptor Reverse Transcriptase (Quiagen, Madrid, Spain). Gene expression was assessed by real time PCR using a LightCycler Thermal Cycler System (Roche Diagnostics, Indianapolis, Indiana, USA). The reaction was performed, following the manufacturer's protocol, in a final volume of 12µl.

The reactions consisted of an initial denaturing of 10 min at 95 ºC, then 40 cycles of 15 seconds denaturing phase at 95ºC, and 1 minute annealing and extension phase at 60ºC. A threshold cycle (Ct value) was obtained for each amplification curve and a ΔCt value was first calculated by subtracting the Ct value for human glyceraldehyde-3-phosphate dehydrogenase cDNA from the Ct value for each sample and transcript. Fold changes compared with the endogenous control were then determined by calculating 2-ΔCt. Every sample was performed in triplicate and negative controls were included in all the reactions. Test reproducibility for all investigated transcripts was less than 0.5% in intertest experiments and even lower in intratest experiments.

3.10 Determination of oxidative stress biomarkers in white blood cells

Oxidative stress biomarkers were analysed in lymphocytes, monocytes and neutrophils using a dual-laser FACSCalibur (Becton Dickinson). Test standardization and data acquisition analysis were performed using the CELL Quest software (Becton Dickinson). For the assessment of ROS generation, including superoxide anion and hydrogen peroxide, cells were incubated with 20.5 µM DCFH-DA (Sigma-Aldrich) at 37°C for 30 min in the

dark and DihidroRhodamine123 5 μM at 37ºC for 30 min (Sigma-Aldrich). For the detection of intracellular GSH, cells were incubated with 1μM 5-chloromethylfluorescein diacetate (CMFDA) (Invitrogen, Eugene, Oregon, USA) for 30 min in the dark at 37ºC. The cells were washed, re-suspended in PBS, and then analysed on a dual-laser FACSCalibur. The JC-1 Mitoscreen assay (BD Biosciences) was used (final concentration 2 μM) to assess Δψm according to manufacturer's instructions.

3.11 Determination of plasma oxidative and nitrosative stress biomarkers

The nitric oxide stable end products nitrite plus nitrate, were measured in plasma from RA patients and healthy donors using a commercial kit (Total Nitric Oxide Assay Kit, Thermo Scientific, Rockford, IL, USA). Plasma total antioxidant capacity (TAC) was analyzed by quantitative colorimetric determination, using TAC Assay Kit (BioVision, Mountain View, CA, USA). Tyrosine nitration, as a marker of nitrative stress, was measured in plasma using Oxiselect Nitrotyrosine ELISA Kit (Cell Biolabs, INC, San Diego, CA, USA) according to manufacturer's instructions.

3.12 Leukocyte activation markers analysis by flow cytometry

Cell surface expression of leukocyte activation markers (CD11b, CD11a, CD62L and CD66b) was detected through flow cytometry. The leukocytes were incubated with specific antibodies at a saturating concentration of 1:10 for 30 minutes in the dark at 4ºC. The antibodies used were: FITC conjugated CD66B (Abcam, Cambridge, UK), PE conjugated CD11B and PE conjugated CD11A (BD pharmigen, Madrid, Spain) and FITC conjugated CD62L (eBioscience, San Diego, CA). Nonspecific antibodies conjugated to FITC and PE were used as negative control. 10000 cells/sample were

acquired in a FASCalibur (Becton-Dickinson) and analyzed by CellQuest software.

The levels of activation markers were determined by the increase of the mean fluorescence intensity (MFI).

3.13 Macrophage differentiation and in vitro treatment with anti-CCPs

THP-1 cell line (ATCC) was cultured in RPMI 1640 medium (Biowest, Nuaillé, France) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), 4 mM L-glutamine (Biowest, Nuaillé, France) and 100 U/ml penicillin, 100 mg/ml streptomycin and 250 pg/ml fungizone (BioWhittaker/MA Bioproducts, Walkersville, Maryland, USA), at 37°C in a humidified 5% carbon dioxide atmosphere.

THP-1 cells ($8x10^5$ cells/ml) were differentiated to macrophages with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 72 hours. The degree of differentiation was confirmed through the increase of autofluorescence and expression of the CD68-FITC marker (eBioscience, Inc, San Diego, CA) measured by flow cytometry using a FASCalibur cytometer.

Differentiated macrophages were incubated with purified anti-CCPs from the pooled sera of 7 RA patients (5 U/ml, 10 U/ml and 20U/ml) (Jackson InmunoResearch Laboratories, Inc, Newmarket, Suffolk, UK) or synthetic human IgG (equal protein concentration that the different anti-CCPs treatments) for 24 h at 37°C.

3.14 Apoptosis assay

Viability was assessed by using an Annexin V-FITC Apoptosis Detection Kit (eBioscience, Bender Medsystems GmbH, Vienna, Austria), according to the manufacturer's recommendations. Cells were trypsinized, washed in PBS and finally dissolved in 195 µl 1x binding buffer and 5 µl of Annexin V, and incubated in darkness for 10 min at room temperature. Cells were then washed with PBS and dissolved in 190 µl in 1x binding buffer and 10 µl (20 µg/ml) propidium iodide (PI). Subsequently, binding of fluorescein-conjugated Annexin V and PI was measured by flow cytometry using a FASCalibur cytometer.

3.15 Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using the statistical software package SPSS (version 15.0 for Windows; SPSS Iberica, Madrid, Spain). Following normality and equality of variance tests, comparisons were made by a parametric test (paired Student's t test) or alternatively by using a non-parametric test (Mann–Whitney rank sum test). Correlations were assessed by Spearman correlation. Differences were considered significant at $p<0.05$.

4. RESULTS

4.1 Clinical and analytical details of RA patients

Table I. Clinical details of the Rheumatoid Arthritis patients and the healthy donors.

	RA patients	Healthy donors
Clinical parameters		
Female/Male (n/n)	53/22	22/9
Age (years)	57.04 ± 11.88	57.87 ± 12.82
Evolution time (years)	12.33 ± 8.93	--------
RF positive (n)	38 (50.66%)	--------
Anti-CCPs antibodies (n)	49 (65.33%)	--------
DAS28	3.49 ± 0.17	--------
Obesity (n)	7 (9.3%) [a]	1(3%)
Diabetes (n)	7 (9.3%) [a]	0
Hypertension (n)	21 (28.0%) [a]	1(3%)
Menopause (n)	25 (33.3%)	9(29%)
Smoker (n)	16 (19.0%)	6 (19%)
CIMT (n)	29 (21.3%) [a]	1 (3%)
Laboratory parameters		
Total Cholesterol, mg/dl	206.20 ± 35.49	201.00 ± 29.92
HDL-Cholesterol, mg/dl	53.96 ± 15.59	59.83 ± 14.88
LDL-Cholesterol, mg/dl	127.34 ± 30.03	124.08 ± 24.59
Triglycerides, mg/dl	122.57 ± 62.85[a]	83.00 ± 40.19
Apolipoprotein A, g/l	146.29 ± 29.33	155.71 ± 29.45
Apolipoprotein B, g/l	88.76 ± 23.37	81.76 ± 19.62
ESR	24.36 ± 1.79 [a]	9.25 ± 1.68
CRP, mg/dl	14.33 ± 2.60[a]	1.15 ± 1.47
Creatinine kinase (U/l)	65.92 ± 33.69	94.1 ± 97.40
C3	136.45 ± 27.03	134.77 ± 49.13
C4	26.56 ± 8.91	26.13 ± 11.96
Treatments		
Corticosteroids	41 (54.6%)	------
Antimalarials (n)	12 (16.0 %)	------
NSAIDS (n)	59 (78.6%)	------
Methotrexate (n)	51 (68.0 %)	------
Anti-TNF treatment	-------	------
Vitamin D	27 (36.0 %)	------

Values are means ± SEM. HDL= High density lipoprotein; LDL= Low density lipoprotein; DAS= Disease activity score; anti-CCPs= Anti-cyclic citrullinated proteins; CIMT= Carotid intima media thickness; ESR= Erythrocyte sedimentation rate; CRP= C reactive protein; NSAIDS= Non-steroideal anti-inflammatory drugs; RF= Rheumatoid factor. [a]Significant differences vs controls ($p < 0.01$).

Clinical details of RA patients and healthy donors included in this study are stated in table I. A 50.66% of RA patients was positive for RF whereas 65.33% presented anti-CCP antibodies. The patients included in the present study had a low-moderate disease activity with a DAS28 of 3.49 ± 0.17, considering moderate level of disease DAS28 values from 2.4 to 3.7 and low level DAS28 < 2.4 (24). C reactive protein (CRP) was significantly increased in RA patients compared to healthy donors. As an early atherosclerotic marker, pathological CIMT was found in 21.3% of the RA patients.

4.2 Altered expression of prothrombotic and inflammatory markers in plasma of RA patients

Compared to healthy donors, RA patients displayed elevated plasma levels of prothrombotic and inflammatory molecules such as Interleukin (IL)-2, IL-6, IL-8, IL-10, IL-17A, IL-23, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), sP-selectine, tumor necrosis factor alpha (TNF-α) and tissue plasminogen activator (tPA) (Supplemental table I).

4.3 Increased oxidative status in leukocytes and plasma RA

Intracellular peroxides and peroxynitrites were significantly increased in monocytes and neutrophils from RA patients compared to healthy donors (Figure 1A). In both cell populations a high percentage of cells with mitochondrial membrane depolarization was further observed (Figure 1B). In contrast, intracellular reduced glutathione was found in both cell types (Figure 1C). In addition, a significant drop in total antioxidant capacity in plasma from RA patients was observed (p=0.002). Nitric oxide levels were lower in plasma from RA patients versus healthy controls

(p=0.049), likely due to its consumption after reacting with anion superoxide and the subsequent formation of peroxynitrite, as suggested by the elevated protein tyrosine nitration in plasma (p=0.046) (Supplemental table I).

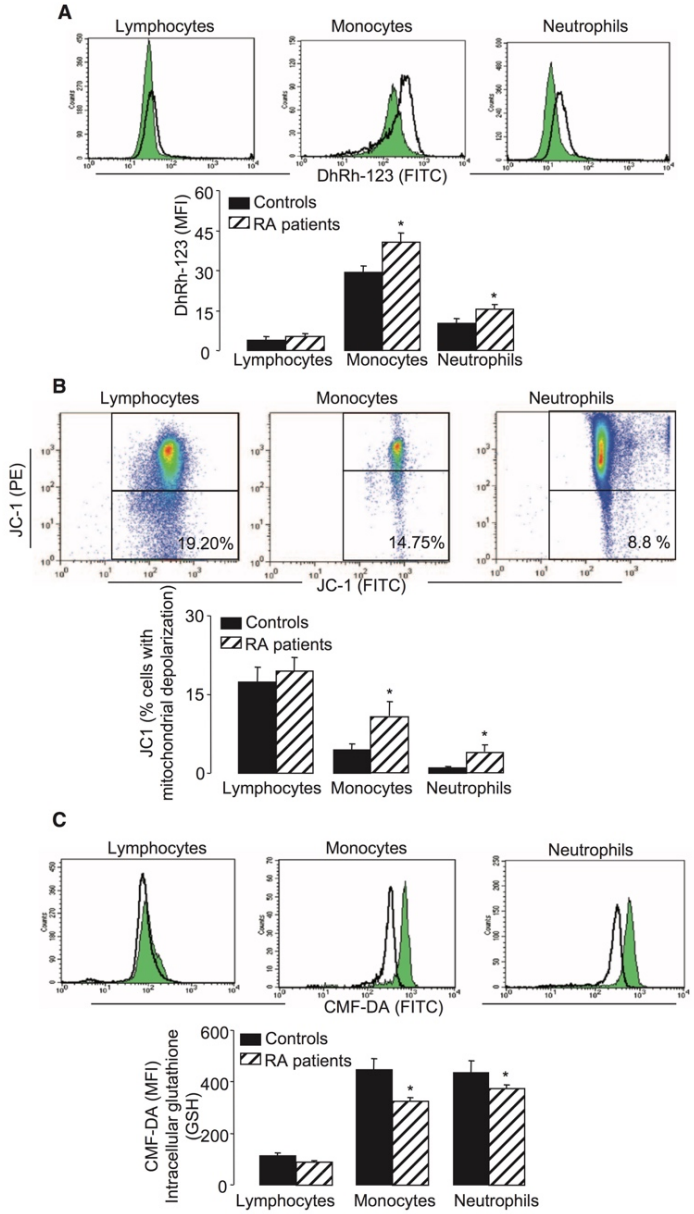

Figure 1. Increased oxidative stress in peripheral monocytes and neutrophils from RA patients. (**A**) Peroxides and peroxynitrites production in neutrophils, monocytes and lymphocytes of RA patients and healthy donors, determined by flow cytometry with the fluorescent probe Dihydrorhodamine-123. (**B**) Proportion of circulating blood cells with

depolarized mitochondria, determined with the JC-1 MitoScreen assay. (**C**) Levels of intracellular glutathione in neutrophils, monocytes and lymphocytes from RA patients and healthy donors, measured through the fluorescent probe CMF-DA. 75 RA and 31 control samples were examined. Representative histograms or dot plots are shown in parallel with bar graphs showing the mean ± SD of median fluorescence intensity (MFI) or percentage of damaged cells of one measurement from 1 sample from each patient and control included in the study. Filled histograms represent controls and empty histograms represent RA patients. (*) indicates significant differences vs healthy donors ($p < 0.05$).

4.4 Correlations and associations among autoantibodies, atherosclerosis, and inflammatory/oxidative stress markers in RA patients

In our cohort of patients with RA, anti-CCPs levels positively correlated with age and DAS28 index (p=0.024 and p=0.001, respectively). A further positive correlation was demonstrated among the anti-CCPs levels and inflammatory plasma markers such as erythrocyte sedimentation rate (ESR)*, C reactive protein (CRP)**, IL-2**, IL-8*, IL-6*, IL-17A**, IL-23**, MCP-1**, MIP-1α**, TNF-α*, MMP-3*, sP-Selectin*, tPA** and VEGF-A** (*p<0.05 and **p<0.01). In relation to oxidative stress, anti-CCP levels positively correlated with tyrosine nitration levels (p=0.032), and negatively with total antioxidant capacity (p=0.002) (Supplemental table II).

Association studies revealed that CMIT was strongly associated with anti-CCP levels (p<0.01) (Supplemental figure I). Additionally, pathological CMIT was associated with increased levels of inflammatory molecules (CRP, MCP-1 and MIP-1α) as well as oxidative stress markers such as decreased antioxidant capacity (Supplemental figure I). These results indicate a direct relationship between oxidative stress and inflammation in the development of atherosclerosis in RA.

No significant correlation was observed between the traditional risk factors (obesity, diabetes and hypertension) and the inflammatory parameters and oxidative markers analyzed in RA patients.

4.5 Differential expression of inflammatory molecules in leukocytes in RA

RT-PCR analysis showed that monocytes were the main contributors to the high levels of IL-1β, TNF-α, MCP-1, TF and IL-8 observed in RA patients (Figure 2A, B, D, E and F), whereas both monocytes and lymphocytes were the source of IL-6 (Figure 2C).

As expected, only lymphocytes expressed IL-17 and IL-2, mRNA, with a great elevation in RA patients vs healthy donors (Figure 2G and H). Even though IL-23 expression was detected in all leukocyte types, only RA lymphocytes showed a significant increase of this cytokine compared to controls (Figure 2L).

Lymphocytes from healthy donors showed higher IL-10 mRNA expression compared to RA lymphocytes whereas RA monocytes had increased IL-10 mRNA expression, although this increment does not seem to account for the high levels of IL-10 found in plasma (figure 2J), suggesting that might be a mechanism of compensation.

Despite MIF-1α was found largely elevated in plasma from RA patients (Supplemental table I), mRNA expression of MIF-α was significantly lower in RA monocytes and lymphocytes vs controls (Figure 2I).

Neutrophils from RA patients displayed high levels of MCP-1 alongside monocytes (Figure 2D). These cells may be also responsible for the elevation of MIP-1α in plasma of RA patients (Figure 2K).

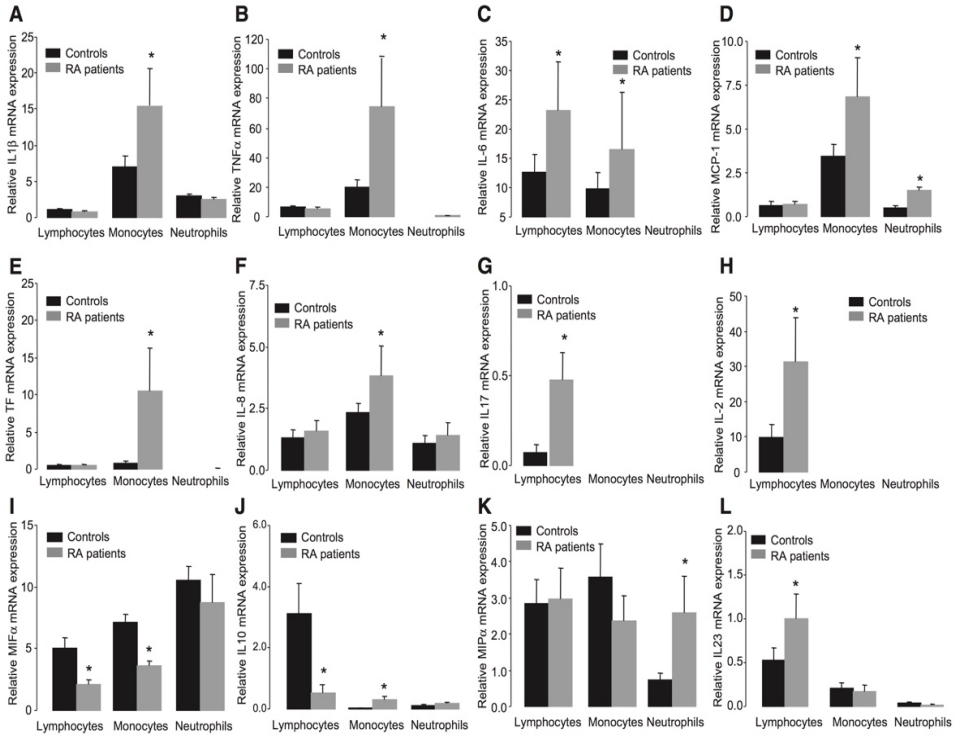

Figure 2. Different mRNA expression profile of inflammatory molecules in RA leukocytes. 75 RA and 31 control samples were examined. (**A-L**) Quantitative real-time RT-PCR was performed on a panel of inflammatory cytokines in lymphocytes, monocytes and neutrophils from each RA patient and healthy donor. (*) indicates significant differences vs healthy donors ($p < 0.05$).

4.6 Leukocyte activation in RA patients

CD11a, CD11b, CD62L and CD66b are well known leukocyte activation markers needed for the adherence to the endothelium and atherogenesis process. In our cohort of RA patients we found a significantly higher expression of CD11b on monocytes and neutrophils ($p<0.01$). RA neutrophils further showed an increase in the cell surface expression of CD11a and CD66b ($p<0.05$) (Supplemental table III).

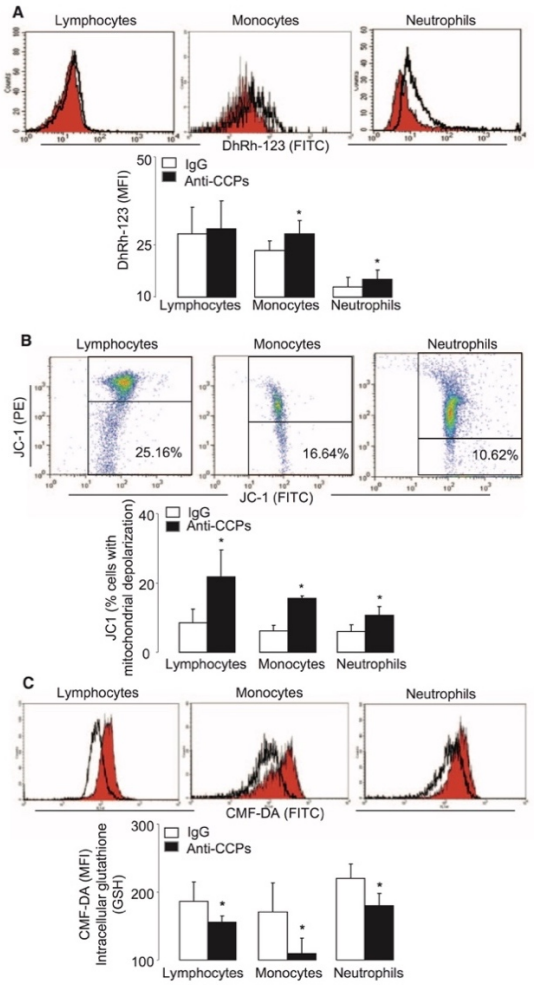

Figure 3. Anti-ccps antibodies induce a pro-oxidative status in healthy leukocytes. Healthy leukocytes were treated with anti-ccps (5 U/ml) or equivalent concentration of synthetic human IgG for 1 hour. Four independent experiments were carried out in samples from 4 different healthy donors (**A**) Peroxides and peroxynitrites production in neutrophils, monocytes and lymphocytes of healthy donors treated with anti-ccps, determined by the fluorescent probe Dihydrorhodamine-123. (**B**) Proportion of circulating blood cells with depolarized mitochondria, determined with the JC-1 MitoScreen assay. (**C**) Levels of intracellular glutathione in neutrophils, monocytes and lymphocytes from healthy donors treated with anti-ccps, measured through the fluorescent probe CMF-DA. Representative histograms or dot plots are shown in parallel with bar graphs showing the mean ± SD of median fluorescence intensity (MFI) or percentage of damaged cells of four independent experiments. Filled histograms represent untreated cells and empty histograms represent leukocytes treated with anti-ccps. (*) indicates significant differences vs cells treated with synthetic human IgG ($p < 0.05$).

4.7 Anti-CCPs antibodies induce a pro-oxidative status in healthy leukocytes

In order to characterize the anti-CCP antibodies, we investigated the IgG subclasses of anti-CCPs isolated from serum of 15 RA patients, having high titers of anti-CCPs and no RF. In accordance with a recent study (25) IgG1 followed by IgG4 were the predominant subclasses among anti-CCPs antibodies (Supplemental figure II).

After 1 hour of treatment with 5 U/ml of anti-CCPs, we found a significant increase in the levels of peroxides in healthy monocytes and neutrophils when comparing to treatment with synthetic IgG (Figure 3A). Additionally, the percentage of cells with depolarized mitochondria significantly rose in all leukocyte subsets after anti-CCPs treatment (Figure 3B). In contrast, addition of anti-CCPs caused a drop in GSH levels (Figure 3C).

4.8 Anti-CCPs antibodies promote a distinct proinflammatory profile in healthy leukocytes

Anti-CCPs significantly increased the mRNA expression of IL-1β, TNF-α, MCP-1, TF and IL-8 in healthy monocytes, in agreement with the results found in vivo in RA patients (Figure 4A, B, D, E and F). Neutrophils also responded to the treatment with anti-CCPs, and an augmentation of IL-1β, MCP-1 and IL-8 was further observed on these cells (Figure 4A, D and F).

In accordance with the vivo studies, anti-CCPs elevated the mRNA expression levels of IL-6 in lymphocytes and monocytes (Figure 4C). It was also noticed a significant increase in IL-2 and IL-23 mRNA levels in lymphocytes after anti-CCPs addition (Figure 4H and L). Alongside lymphocytes, neutrophils were the cell type that expressed higher levels

of IL-23 in response to anti-CCPs treatment (Figure 4L). In agreement with the in vivo studies, in vitro anti-CCPs treatment elevated the expression of MIP-1α mRNA in healthy neutrophils, confirming that anti-CCPs antibodies are the responsible for the augmented MIP-1α expression in neutrophils and its elevation in plasma from RA patients (Figure 4K).

In contrast to that observed in RA patients, no changes in IL-17 expression were observed between IgG and anti-CCPs treatments in lymphocytes (data no shown). Although no differences were seen in the expression of VEGF and its receptor, VEGFR1, comparing leukocytes from controls and RA patients (data not shown), we detected a significant increase of these proteins in monocytes and neutrophils after anti-CCPs treatment (Figure 4I and J). Finally, the altered expression of IL-10 in RA monocytes and lymphocytes was fully recapitulated in these cell types after treatment with anti-CCPs (Figure 4G).

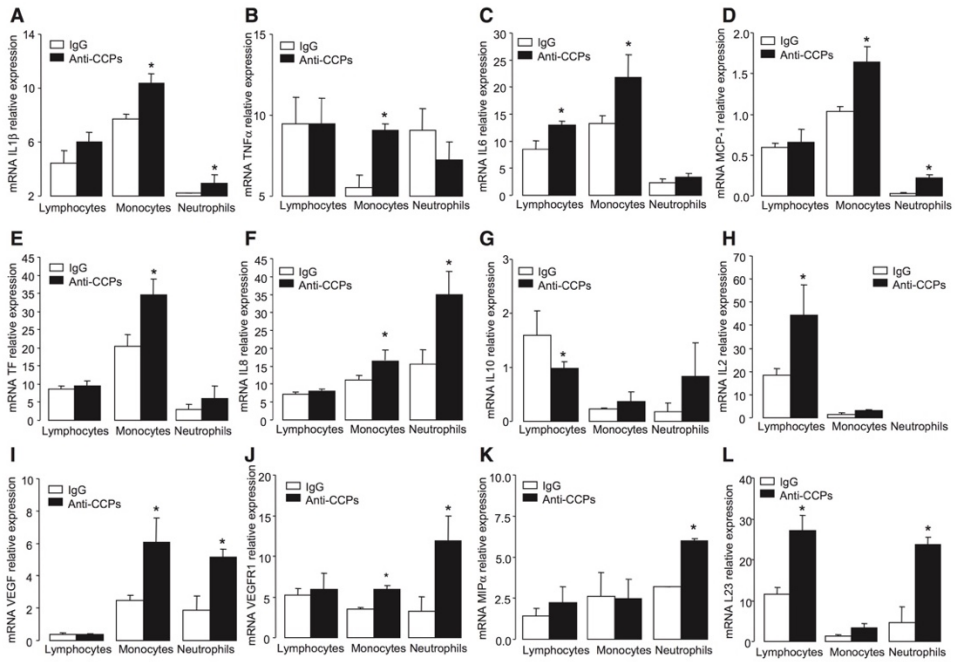

Figure 4. Anti-CCPs antibodies promote a distinct proinflammatory profile in healthy leukocytes. Four independent experiments were carried out in samples from 4 different

healthy donors (**A-L**) Quantitative real-time RT-PCR was performed on a panel of inflammatory cytokines in lymphocytes, monocytes and neutrophils from healthy donors treated with anti-ccps or equivalent concentration of synthetic human IgG for 6 hours. (*) indicates significant differences vs cells treated with synthetic human IgG ($p < 0.05$).

In order to further validate the specificity of the anti-CCP antibodies, healthy lymphocytes, monocytes and neutrophils were treated with total IgGs from 3 RA patients depleted or non-depleted in anti-CCPs, IgGs from pooled sera of 4 healthy donors, IgGs from pooled sera of 4 patients with ankylosing spondylitis (AS) (non autoimmune inflammatory disease) and a commercial monoclonal anti-CCP (anti-citrullinated fibrinogen). The depletion of anti-CCPs activity in IgGs-RA patients was 40-70%, depending on the initial activity observed in each RA sample, probably due to the excess of column binding capacity (Supplemental figure III).

The results showed that the increased expression of genes induced by isolated anti-CCPs (through ELISA method) was recapitulated when treating healthy leukocytes with total IgGs from RA patients. Thus, mRNA expression of MIP-1α, IL-23 and MCP-1 was increased in neutrophils, IL-2 and IL-23 was elevated in lymphocytes and IL-1β, MCP-1, IL-6, TF and VEGF was upregulated in monocytes after treatment with 500 ug RA IgGs (Figure 5). To demonstrate the specificity of anti-CCPs, leukocytes were treated with RA IgGs depleted in anti-CCPs. A significant reduction of the mRNA expression of these genes was observed (Figure 5).

Moreover, the monoclonal anti-CCP promoted a significant elevation of the expression of all the genes evaluated. In the other hand, no effects in the expression of these genes were noticed after treatment with IgGs from AS patients, indicating the role of autoimmune system in the pathology of RA.

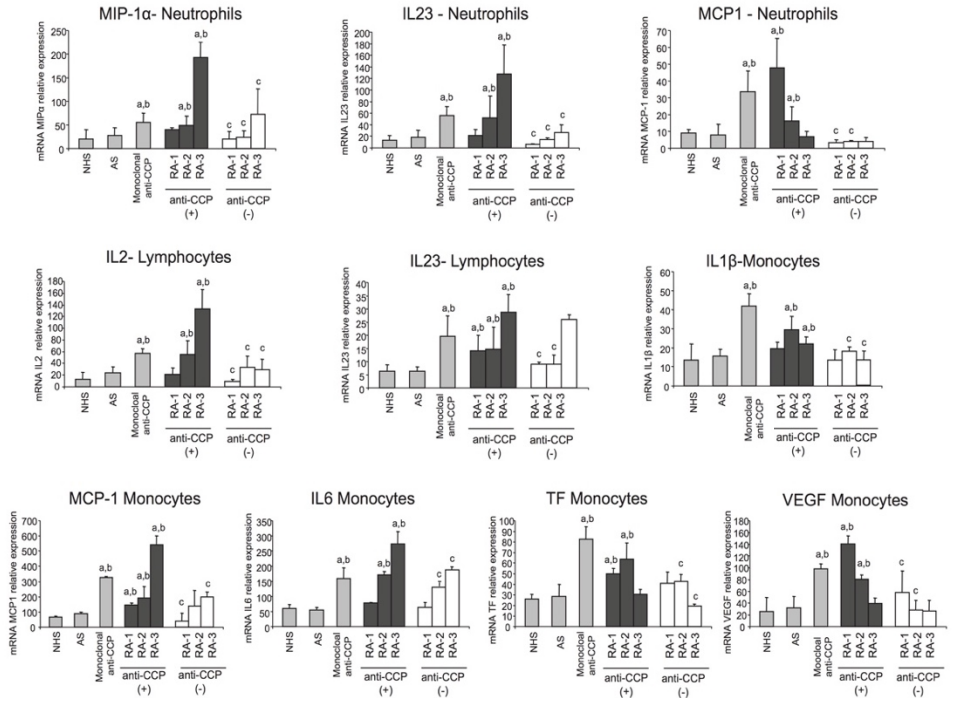

Figure 5. Anti-CCPs specifically induce the expression of inflammatory and pro-thrombotic molecules in white blood cells. Healthy neutrophils, lymphocytes and monocytes were treated with 500 μg IgGs from 3 RA patients depleted or non-depleted in anti-CCPs, IgGs from AS patients, IgGs from healthy donors and 10 μg/ml monoclonal anti-CCP (anti-citrullinated fibrinogen IgG) for 6 hours. Prior to the different treatments, cells were treated with human anti-FCR to block the non-specific bindings for 30 minutes. Panels show the mean ± SEM of two separated experiments performed in duplicate. (a) indicates significant differences vs cells treated with normal human serum IgGs ($p < 0.05$), (b) indicates significant differences vs cells treated with ankylosing spondylitis serum IgGs ($p < 0.05$) and (c) indicates significant differences vs cells treated with RA IgG non-depleted in anti-CCPs($p < 0.05$).

4.9 Anti-CCPs antibodies increase the expression of leukocyte activation markers

After 6h treatment with anti-CCPs, a significant elevation of CD11b, and CD66b was observed in neutrophils (p=0.019 and p=0.045, respectively) and an increase of CD11a was detected in monocytes (p=0.010) (Supplemental table IV). These results indicated that anti-CCPs are directly responsible for leukocyte activation, and might therefore be involved in

the vascular complications and the development of atherosclerosis associated to RA.

4.10 Anti-CCPs antibodies induce apoptosis and impair macrophage function

Anti-CCPs treatment during 24h induced apoptosis in THP-1 derived macrophages in a dose-dependent manner, as demonstrated by changes in cell morphology (Supplemental figure IV-A) and the increased percentage of Annexin V/PI positive cells (11% and 19% of apoptosis after 10U/ml and 20U/ml anti-CCPs treatment, respectively) (Supplemental figure IV-B). These results were further supported by the increased mRNA expression of molecules involved in apoptosis, including Fas cell surface death receptor (FAS), BCL2-Interacting Killer (BIK), Tumor Protein P53 (TP53) and B-Cell CLL/Lymphoma 2 (BCL2) (Supplemental figure 3C). Effects in the macrophage inflammatory state were also noticed: treatment with anti-CCPs increased the mRNA expression of cytokines such as IL-10, IL-23, TNFα and IL-1β in a dose-dependent manner (Supplemental figure IV C).

5. DISCUSSION

Anti-CCPs are highly specific predictive and diagnostic markers of RA (26-28), and they have been found to be pathogenic in experimental murine models of arthritis (29, 30). In humans, there is a close relationship between anti-CCP levels and inflammation, oxidative stress and atherosclerosis associated to RA (31). Nevertheless, the direct effects of these auto-antibodies in the function of the human leukocytes and macrophages have not been studied yet. Here we show for the first time, that anti-CCP antibodies act as direct inductors of the pro-oxidative status,

inflammatory and atherogenic profile of lymphocytes, monocytes and neutrophils in the CVD associated to RA. In addition, effects on macrophage activation and alteration of its apoptotic programme are also reported.

Oxidative stress has been described in RA and growing evidence suggests it could be used for earlier diagnosis of RA (32). Here, we confirm the increased oxidative stress and impaired antioxidant capacity in RA both at plasma and cellular levels. In our cohort, RA plasma had high levels of oxidative stress markers and diminished levels of antioxidant capacity. These results are in agreement with recent studies showing a pro-oxidative status and impairment of antioxidant capacity in RA, measured as altered levels of MDA (malondialdehyde), GSH, GSH Px (GSH peroxidase), GAH reductase, superoxide dismutase, catalase, glucose-6-phosphate, vitamin E and total NO (32, 33).

Although studies at plasma levels are well defined, the investigations at cellular level are more limited. In our study, neutrophils and monocytes from RA patients showed mitochondrial depolarization, increased ROS and peroxynitrite levels and lower levels of GSH. These results agree with a recent study showing enhanced production of superoxide and peroxynitrites in monocytes and neutrophils of RA patients (34). Interestingly, in our cohort of patients, no changes in oxidative stress were seen in lymphocytes. Yet, other authors have described an alteration in mitochondria polarization and cell surface-thiols in lymphocytes from RA patients (35, 36). This could be due to the different disease score or the percentage of patients with high anti-CCP titers. Our data was further supported by in vitro studies, demonstrating the direct effect of anti-CCPs in the induction of an oxidative status in monocytes and neutrophils.

The association between oxidative stress and anti-CCPs was also validated by a recent study showing that a deletion polymorphism in glutathione S-transferase Mu-1 (antioxidant enzyme) was associated with anti-CCP positivity (37), suggesting that oxidative stress might be involved in the development of RA-specific autoimmunity in genetically susceptible subjects. Moreover, a recent study showed an increased synovial oxidant activity in anti-CCP positive RA patients compared to RA patients negative for anti-CCPs (38).

It is well established that cytokine networks play a critical role in the pathogenesis of RA. There is an elevation of proinflammatory cytokines in RA plasma and synovial fluid (reviewed in 39). Accordingly, our cohort of RA patients displayed elevated plasma levels of proinflammatory cytokines which, in addition, strongly correlated with elevated levels of anti-CCPs in plasma, suggesting a role for these autoantibodies in the inflammatory profile associated with RA. In support for this notion, there is evidence of a different cytokine profile associated with anti-CCP or rheumatoid factor antibodies (40). Moreover, it has been shown that anti-CCP positive patients had significant higher levels of IL-1β, IL-10 and IL-17 in their synovial fluid than anti-CCP negative patients (41), which is in line with our data in plasma from RA patients.

Searching the source of these proinflammatory molecules, we observed a different profile for each cell type: lymphocytes, monocytes and neutrophils. RA lymphocytes particularly expressed elevated levels of IL17, IL2, IL6 and IL23, and decreased levels of IL-10. Interestingly, in vitro treatment with anti-CCPs fairly recapitulated the proinflammatory profile

seen in RA lymphocytes, increasing the expression of IL2, IL6 and IL23, and lowering the expression of IL-10.

The role of these cytokines has extensively been described in the development of different T cell subsets in RA such as Th1, Th17 and Treg cells (39). Our study suggests that anti-CCps might promote the differentiation of Th1 and the impairment of Treg cells, main characteristics of the physiopathology of RA. IL-17 is a newly identified cytokine associated with the pathogenesis of RA and describing the specific cell subtype: Th17. Number of Th17 cells is increased in peripheral blood of RA patients, and IL-17, which is greatly released in plasma and synovial fluid, has relevant roles mediating inflammation, microvascular function and atherosclerosis (42-44). In our hands, although IL-17 levels strongly correlated with anti-CCP levels, in vitro treatment of normal lymphocytes with anti-CCPs did not induce its expression. In this regard, it has been demonstrated that monocytes and fibroblasts from inflamed joints are able to induce the development of Th17 cells (45, 46), suggesting that interactions with other activated cells might be further required to induce the expression of IL17.

Despite RA is recognized as T helper-driven disease, monocytes/macrophages are important effectors in its pathology, being central players in inflammation and atherosclerosis. In our cohort of RA patients, a significant rise in IL-1β, TNFα, IL6, MCP1, IL-8 and tissue factor mRNA was observed in peripheral monocytes. Of note, in vitro anti-CCPs treatment of healthy monocytes and THP-1 derived macrophages fully recapitulated this procoagulant and inflammatory profile. Additionally, this aspect further supported by the direct relationship found in vivo between anti-CCP titers and levels of inflammatory molecules.

Macrophages plasticity plays a central role during the development of RA (47). Anti-CCPs treatment promoted the expression of inflammatory cytokines typically associated to the classical inflammatory macrophage M1 state. This data points to anti-CCP antibodies as inductors of the M1 polarization in RA. Interestingly, increasing anti-CCP doses promoted apoptosis in macrophages. Accumulating evidence indicates that impaired apoptosis represents a mechanism underlying the pathogenesis of RA. Although apoptotic cells are rarely found in RA tissues in vivo, diverse RA cells have often been observed to express high levels of FAS and are highly susceptible to apoptosis in vitro, likely reflecting multiple anti-apoptotic processes or alteration in the normal apoptosis programme in RA (48). In that way, elevated levels of FAS mRNA and other pro/anti-apoptotic molecules (p53, BIK and Bcl-2) was shown after treatment with anti-CCPs, suggesting that the alteration in the apoptosis process in RA could be due to the action of anti-CCP antibodies.

Neutrophils play a relevant role in RA, being the predominant cells in synovial fluid. MIP-1α is highly expressed by RA neutrophils from synovial fluid, contributing to the recruitment of mononuclear cells from the bloodstream into synovium (49). According to previous evidence, we found high MIP-1α levels in plasma from RA patients (50). Adding new data about the source of this molecule in plasma, we show that RA neutrophils were the cells expressing highest MIP-1α mRNA levels compared to healthy donors, indicating that they are the main responsible for elevated blood MIP-1α levels in RA. Moreover, it was observed a strong correlation between MIP-1α and anti-CCP levels, indicating that anti-CCPs are directly involved in the production of this chemokine. We further confirm this hypothesis showing that anti-CCPs significantly

increased levels of MIP-1α in healthy neutrophils, while no changes were seen in monocytes and lymphocytes. Neutrophils have been shown to produce a large variety of proinflammatory cytokines as well as pro-resolving mediators. In addition, the direct interaction of neutrophils with macrophages, dendritic cells, natural killer cells, and lymphocytes modulates the immune response (51). In that way, we could demonstrate that treatment of neutrophils with anti-CCPs induced in the expression of higher number of inflammatory molecules than those observed in vivo in RA neutrophils. These data further suggest the relevant role of anti-CCPs as inductors of an inflammatory profile in neutrophils.

The prevalence of CVD in RA patients is well documented and anti-CCP antibodies have been linked to an adverse cardiovascular profile (31). In agreement with the existing body of literature, we found altered diverse parameters strongly associated with the development of atherosclerosis in RA patients. Thus, an increased presence of activation markers in neutrophils and monocytes and elevated expression of TF in monocytes were seen in RA patients. Additionally, 21.3% RA patients had pathological CMIT and there was a strong association between pathological CMIT and DAS28, CRP levels, MCP-1 plasma, MIP-1alpha levels and low TAC and anti-CCP levels. These results are in agreement with a study indicating that anti-CCP positive RA patients had higher CIMT than patients without evidence of these antibodies (52). Moreover, it has been shown that citrullinated proteins are prevalent within atherosclerotic plaques, and certain anti-CCP antibodies are correlated with the CMIT (53) and associated with the atherosclerotic burden (54). In this sense, treatment of healthy leukocytes with anti-CCPs resulted in an elevation of cell activation markers in monocytes and neutrophils, an increase of TF

expression, inflammation and oxidative stress, indicating the participation of these autoantibodies in the development of atherosclerosis and CVD. Although anti-CCP-positive and anti-CCP-negative patients display similar clinical features, they have a different disease course, being more severe in anti-CCP positive RA patients. Here we demonstrate that anti-CCP antibodies may directly increase the risk of cardiovascular disease in RA. This was confirmed through both, the associations among anti-CCP levels and parameters related to inflammation, oxidative stress and atherosclerosis in vivo and the in vitro treatment of healthy leukocytes with anti-CCPs. Thus, anti-CCPs activate leukocytes and change their phenotype to an atherogenic profile through the: a) alteration of the oxidative status in monocytes and neutrophils, b) promotion of the T helper 1 differentiation and the impairment of regulatory T cells, c) modulation of the MIP-1α expression in neutrophils, increasing the migratory properties of mononuclear cells, d) elevation of the inflammatory and procoagulant monocyte activity and e) the polarization of macrophages to a classical inflammatory M1 state and alteration of the apoptosis programme in macrophages.

Targeting these autoantibodies would be an excellent strategy to prevent the development of CVD associated with RA.

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7. SIGNIFICANCE

This is a novel detailed study which identifies the specific role of anti-CCPs in the induction of the pro-inflammatory and pro-atherogenic profile in RA patients.

Specifically, the key novel findings are:

The identification of lymphocytes as mayor players in altered inflammatory profile, monocytes in the prothrombotic and atherogenic status, and neutrophils in a pro-oxidative feature, highlights the relevance of each cell type in the RA physiopathology.

In vitro treatment of healthy monocytes, lymphocytes and neutrophils with anti-CCPs purified from clinical samples fully recapitulated the pathogenic profile observed in leukocytes from RA patients, promoting their activation and changing their phenotype to an atherogenic profile. Thus, our study stresses the importance of anti-CCP antibodies in the pathogenesis of RA and specifically in the onset of atherosclerosis. Targeting the mechanisms involved would be an excellent strategy to prevent the development of cardiovascular disease related to RA.

8. SUPPLEMENTARY INFORMATION

Supplemental table I. Thrombotic/inflammatory factors and oxidative/nitrosative stress parameters altered in plasma from RA patients vs healthy donors.

	RA patients	Healthy donors	Statistical significance
VEGF-A, pg/ml	1124.56 ± 345.30	361.85 ± 77.11	p=0.206
sCD40L, ng/ml	17356.32 ± 2554.61	21208.04 ± 4509.47	p=0.440
IFNγ, pg/ml	568.18 ± 336.71	6.15 ± 3.30	p=0.111
IL-2, pg/ml	180.70 ± 76.39*	0.00 ± 0.00	p=0.029
IL-6, pg/ml	18.12 ± 8.39*	0.00 ± 0.00	p=0.044
IL-8, pg/ml	44.25 ± 8.23**	13.89 ± 7.53	p=0.009
IL-10, pg/ml	33.38 ± 13.31*	0.28 ± 0.26	p=0.016
IL-17A, pg/ml	194.22 ± 83.54*	0.00 ± 0.00	p=0.031
IL-23, pg/ml	1761.36 ± 912.60*	0.00 ± 0.00	p=0.045
MCP-1, pg/ml	1048.69 ± 74.83**	548.79 ± 41.64	p=0.000
MIP-1alpha, pg/ml	1149.68 ± 235.12**	340.66 ± 133.65	p=0.004
MMP-13, ng/ml	0.27 ± 0.03	0.18 ± 0.04	p=0.123
sP-Selectin, ng/ml	280.08 ± 19.63**	198.66 ± 21.34	p=0.009
TNFα, pg/ml	751.50 ± 491.78*	0.00 ± 0.00	p=0.048
tPA, pg/ml	5695.93 ± 1016.72*	1770.54 ± 233.86	p=0.046
Total antioxidant capacity (TAC, mM Trolox equiv)	87.64 ± 1.97**	128.59 ± 13.03	p=0.002
Nitric oxide, μM	11.95 ± 1.32*	18.99 ± 3.60	p= 0.049
N-Tyr, nM (Protein tyrosine nitration)	94.60 ± 30.65*	21.83 ± 4.58	p= 0.046

Values are means ± SEM. VEGF-A, Vascular endothelial growth factor A; IFN-γ, Interferon gamma; MCP-1, Monocyte chemotactic protein 1; MMP-13, Metaloprotease 13 ; MIP-1alpha , macrophage inflammatory protein 1 alpha; TNF-α, Tumor necrosis factor alpha ; tPA, Tissue plasminogen activator. Significant differences vs healthy donors *p <0.05 and **p<0.01

Supplemental table II. Correlations among anti-CCP antibody titers and inflammation/oxidative stress markers in RA patients.

	Anti-CCPs (UI)	
	Rho-Spearman	P
Age	0.227	0.024
DAS	0.508	0.001
CRP (mg/dl)	0.364	0.001
ESR	0.289	0.008
IL-2 (pg/ml)	0.481	0.002
IL-8 (pg/ml)	0.312	0.013
IL-6 (pg/ml)	0.357	0.024
IL-17A (pg/ml)	0.482	0.002
IL-23 (pg/ml)	0.479	0.002
MCP-1 (pg/ml)	0.465	0.001
MIP-1alpha (pg/ml)	0.363	0.005
TNFα(pg/ml)	0.401	0.010
MMP-13 (ng/ml)	0.379	0.025
sP- Selectin (ng/ml)	0.359	0.034
tPA (pg/ml)	0.444	0.001
VEGF-A (pg/ml)	0.413	0.001
N-Tyr (nM)	0.331	0.032
TAC (mM Trolox equiv)	-0.394	0.002

Anti-CCPs= anti-cyclic citrullinated proteins; DAS= disease activity score; CRP= C reactive protein; ESR= erythrocyte sedimentation rate; MCP-1= monocyte chemotactic protein 1; MIP-1α= macrophage inflammatory protein 1 alpha; TNFα= tumor necrosis factor alpha; tPA= tissue plasminogen activator; VEGF= vascular endothelial grow factor A; N-Tyr= tyrosine nitration; TAC= total antioxidant capacity

Supplemental table III. Activation of leukocytes in RA patients

	RA patients	Healthy donors	Statistical significance
CD11a			
Lymphocytes	173.95 ± 13.64	175.19 ± 29.55	p=0.970
Monocytes	165.88 ± 11.75	180.55 ± 32.94	p=0.680
Neutrophils	65.55 ± 3.43*	45.58 ± 6.66	p=0.016
CD11b			
Lymphocytes	18.11 ± 1.86	15.17 ± 3.02	p=0.417
Monocytes	56.86 ± 4.99*	29.95 ± 4.79	p=0.019
Neutrophils	53.41 ± 4.43**	30.75 ± 5.98	p=0.005
CD62L			
Lymphocytes	15.58 ± 1.02	16.81 ± 2.95	p=0.699
Monocytes	22.90 ± 1.33	20.26 ± 3.44	p=0.486
Neutrophils	30.69 ± 1.70	27.47 ± 4.38	p=0.503
CD66b			
Lymphocytes	2.63 ± 0.41	4.62 ± 1.38	p=0.188
Monocytes	5.81 ± 0.60	6.01 ± 0.71	p=0.830
Neutrophils	19.79 ± 1.42*	15.34 ± 1.52	p=0.039

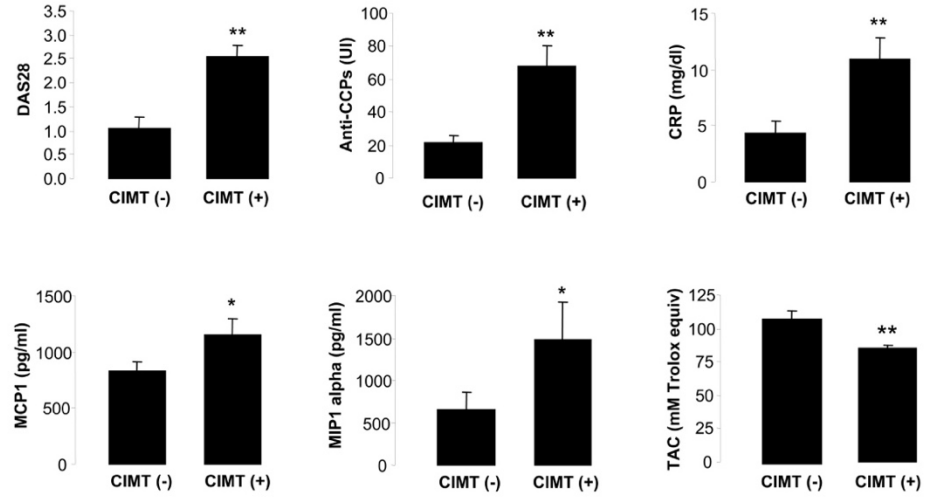
Values are means ± SEM. CD11a, Cluster of differentiation antigen 11a; CD11b, Cluster of differentiation 11b; CD62L, Cluster of differentiation 62L, L-selectin; CD66b, Cluster of differentiation 66b. Significant differences vs controls *p <0.05 and **p<0.01

Supplemental table IV. Markers of leukocyte activation in healthy leukocytes after treatment with anti-CCPs isolated from RA patients

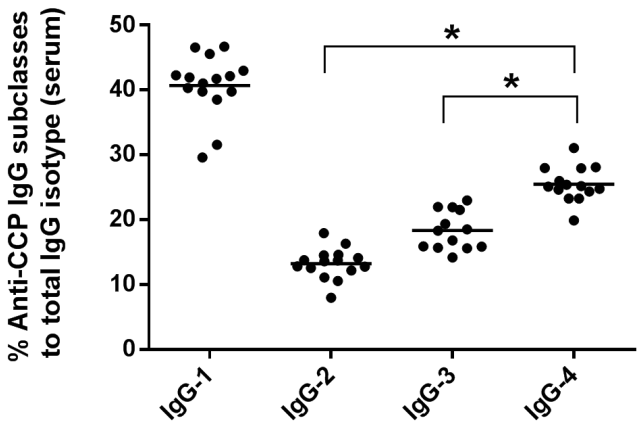
	IgG	Anti-CCPs	Statistical significance
CD11a			
Lymphocytes	41.81 ± 4.51	45.71 ± 6.92	p=0.661
Monocytes	55.33 ± 0.13	61.56 ± 0.81*	p=0.010
Neutrophils	24.94 ± 1.23	31.03 ± 2.11	p=0.085
CD11b			
Lymphocytes	35.85 ± 6.10	42.56 ± 5.83	p=0.471
Monocytes	14.04 ± 5.33	19.93 ± 4.97	p=0.499
Neutrophils	74.83 ± 17.20	149.91 ± 16.12*	p=0.019
CD62L			
Lymphocytes	49.99 ± 4.14	51.28 ± 7.12	p=0.677
Monocytes	23.36 ± 0.85	24.09 ± 0.94	p=0.599
Neutrophils	15.30 ± 1.54	12.16 ± 1.11	p=0.135
CD66b			
Lymphocytes	23.07 ± 0.89	24.13 ± 2.19	p=0.883
Monocytes	18.91 ± 0.84	16.70 ± 1.15	p=0.198
Neutrophils	88.64 ± 11.51	147.39 ± 23.86*	p=0.045

Values are means ± SEM. CD11a, Cluster of differentiation antigen 11a; CD11b, Cluster of differentiation 11b; CD62L, Cluster of differentiation 62L, L-selectine; CD66b, Cluster of differentiation 66b. Significant differences vs IgG control *p <0.05.

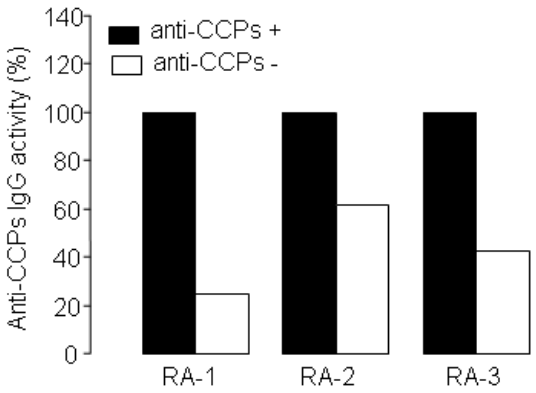
Supplemental figures



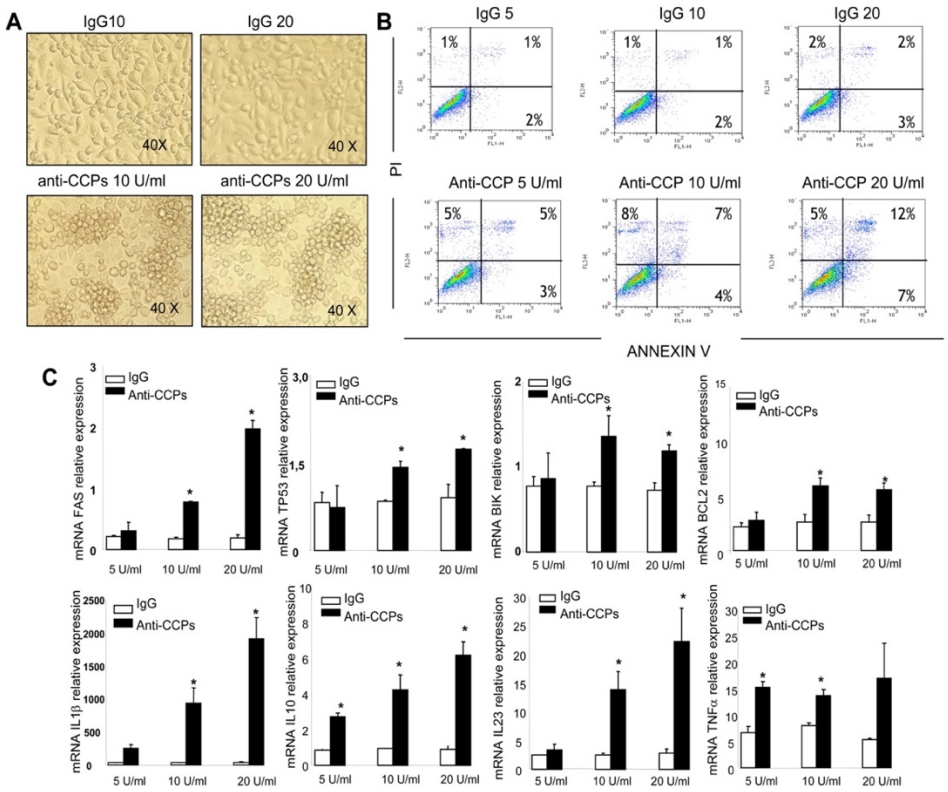
Supplemental figure I. Associations among carotid intima media thickness and parameters of autoimmunity, inflammation and oxidative stress in RA patients. Comparisons were made by a parametric test (paired Student's t test) *p<0.05 vs normal CMIT. **P<0.01 vs normal CMIT.



Supplemental figure II. Following IgG1, IgG4 are the most predominant IgG subclass among anti-CCPs antibodies. Anti-CCPs antibodies were isolated from 15 RA patients with high titres of anti-CCPs. Dot plot shows the % of each IgG subtype in each RA patient. *p<0.05 vs IgG2 and IgG3.



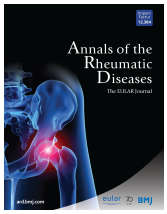
Supplemental figure III. Affinity depletion of anti-CCP antibodies from RA patients. IgGs from serum of 3 RA patients with high titers of anti-CCPs were isolated using HiTrap protein G HP columns. Anti-CCPs from IgGs-RA patients were purified using Streptavidin agarose columns coupled with biotinylated CCP peptides. The IgGs-containing anti-CCPs from RA serum were incubated with the CCP affinity column 1 hour at room temperature. After incubating the sample the column was washed with PBS to obtain the flow through with IgGs depleted in anti-CCPs. Anti-CCP-IgG activity of starting material (purified IgGs) and flow-through (IgGs depleted in anti-CCPs) was measured by ELISA (Eurodiagnostica).



Supplemental figure IV. Anti-CCPs induce apoptosis and increased expression of inflammatory cytokines in macrophages**.** THP-1 cell line was differentiated to macrophages using PMA (100 ng/ml) for 72 hours. Thereafter, cells were treated with increased doses of anti-CCPs or equivalent concentration of synthetic human IgG for 24 hours. (**A**) Phase microscopy with a magnification of 40X (**B**) Apoptosis was measured through flow cytometry using Annexin V-FITC conjugated and propidium iodide (PI) (C) Quantitative real-time RT-PCR was performed on a panel of molecules involved in apoptosis and inflammatory cytokines. (*) indicates significant differences vs cells treated with synthetic human IgG ($p < 0.05$).

CAPÍTULO II

EXTENDED REPORT



Gene profiling reveals specific molecular pathways in the pathogenesis of atherosclerosis and cardiovascular disease in antiphospholipid syndrome, systemic lupus erythematosus and antiphospholipid syndrome with lupus

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Gene profiling reveals specific molecular pathways in the pathogenesis of atherosclerosis and cardiovascular disease in Antiphospholipid syndrome, Systemic Lupus Erythematosus and Antiphospholipid Syndrome with Lupus

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* C P-S and N.B. shared first authorship.

MAA and Ch L-P shared last authorship.

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Keywords. Antiphospholipid Syndrome, Systemic lupus erythematosus, atherosclerosis, cardiovascular disease, gene profile.

1. ABSTRACT

Objective: To identify shared and differential molecular pathways involved in the pathogenesis of atherosclerosis (AT) and cardiovascular disease (CVD) in Systemic Lupus Erythematosus (SLE), primary Antiphospholipid syndrome (APS) and APS associated with SLE (APS plus SLE).

Methods: 129 patients (42 APS, 31 APS plus SLE and 56 SLE) and 61 healthy donors were included. Microarray expression profiling was performed in monocytes. RT-PCR of selected genes and Western blot were used to validate microarray data. Clinical and inflammatory parameters were also analyzed.

Results: Comparing to controls, 555, 1224, and 518 genes were differentially expressed in monocytes from SLE, APS plus SLE, and APS patients, respectively. Approximately 25-30% of differentially expressed genes were related to AT and CVD. Each disease displayed a specific AT/CVD/Inflammation-related gene signature. Compared to SLE, APS showed alterations in mitochondria biogenesis and function and oxidative stress. Besides the interferon signature, found in APS plus SLE and SLE patients, various genes mediating atherosclerotic/inflammatory signalling were also differentially expressed in APS plus SLE. IgG-aCL titers independently predicted both atherosclerotic and thrombosis in APS plus SLE. Moreover, a significant correlation of IgG-aCL titers with mRNA levels of certain inflammatory molecules in monocytes was further noticed. In vitro treatment of monocytes with IgG-aCL promoted an increase in the expression of the genes most significantly changed in APS plus SLE vs healthy donors.

Conclusion: Gene expression profiling allowed the segregation of APS, APS plus SLE and SLE, with specific signatures explaining the pro-atherosclerotic and pro-thrombotic alterations in these highly related autoimmune diseases.

2. INTRODUCTION

The development of atherosclerosis and cardiovascular disease in Antiphospholipid syndrome (APS) and systemic lupus erythematosus (SLE) involves genetic factors as well as other acquired and modifiable risk factors (e.g., hypercholesterolemia, diabetes mellitus and hypertension). Inflammatory components of the immune response, as well as autoimmune elements (e.g., autoantibodies, autoantigens and autoreactive lymphocytes) seem to be also involved in these processes. In addition, oxidative stress as well as dyslipidemia and various systemic inflammation mediators, including cytokines, chemokines, prothrombotic molecules, and adhesion receptors, among others, have been implicated in the development of these vascular pathologies[1,2]. APS and SLE share several clinical and molecular features, but also have some unique distinguishing characteristics. Thus, antiphospholipid antibodies (aPLs) and other autoantibodies are responsible for the development of atherothrombosis in APS. aPLs promote the overexpression of tissue factor (TF) and protease activated receptors (PARs)[3-5]. They are also responsible for the altered protein profile of monocytes and related to thrombosis development including overexpression of annexins I and II or RhoA proteins among others[6]. Besides, aPLs promote oxidative perturbations and mitochondrial dysfunction[7-9] and also trigger an inflammatory cascade with increased expression of several cytokines, chemokines and mediators of endothelial dysfunction[10,11]. Furthermore,

aPLs cross-react with oxidized low-density lipoproteins (ox-LDLs), thus accelerating their influx into macrophages, and promoting monocyte activation and atherosclerosis development[12]. Moreover, serum levels of anti-cardiolipin (aCL) antibodies correlate with the incidence and severity of acute coronary syndrome, myocardial infarction, and stroke[13,14].

Relevant factors directly influencing the development of atherosclerosis and CVD in SLE comprise drug therapy, immune complex generation and changes in innate immune responses, complement activation, endothelial dysfunction, oxidative stress, increased production of adipokines, dysfunctional lipids, and changes in the production and activity of a complex network of cytokines[15,16].

APS associated with SLE (APS plus SLE or SAPS) is diagnosed in patients having polyautoimmunity, that is, who have SLE and have also suffered thrombotic events or adverse pregnancy outcomes in the presence of aPLs[17]. Although APS plus SLE and APS patients have similar clinical profiles, heart valve disease, haemolytic anemia, low C4 levels, and neutropenia seem to be more common in patients with APS plus SLE [18]. All that specific characteristics of those three pathologies have significant implications for the therapy. Therefore, it is of great relevance to clearly define each clinical entity and evaluate the patient based upon a complete knowledge of the underlying disease processes.

The pathogenesis of atherosclerosis involves various cell types of the immune system, having been shown that monocytes play an essential role. In addition to modulate lipid metabolism, monocytes secrete inflammatory cytokines, chemokines and reactive oxygen species that drive the pathogenesis. They also produce TF and proteases that contribute to thrombosis and atherosclerotic plaque rupture[19,20].

Characterizing the monocytes molecular signature may provide an alternative and effective way to identify heterogeneous autoimmune patient's subpopulations for targeted therapies. Microarray analysis is a broad-based profiling method that permits the concomitant comparison of gene expression profiles among different study groups, revealing active networks of interrelated genes within subpopulations under study[21].

Few microarray studies in autoimmunity have been reported, and only some published reports have described results obtained using microarray analysis of peripheral blood mononuclear cells populations from patients with SLE[22-25]. Yet, none of them have performed comparative studies in purified monocytes from APS, APS plus SLE, and SLE.

Thus, by using microarray technology in combination with other protein analyses, the present study intended to identify shared and differential molecular pathways involved in the pathogenesis of atherosclerosis and CVD in these autoimmune disorders.

3. PATIENTS AND METHODS

One hundred and twenty six patients, forty one with APS, thirty one with APS plus SLE and fifty four with SLE, as well as sixty one healthy donors were included in the study (during a period of 24 months) after ethics committee approval was obtained. Subjects were selected among patients with stable disease for more than six months, without infections, abortions, thrombosis, or changes in their treatment protocol. All patients provided written informed consent. None of the healthy controls had a history of autoimmune disease, bleeding disorders, thrombosis, or pregnancy loss. The characteristics of the patients and the controls are shown in Table I.

Table I. Clinical and laboratory parameters for APS, APS+SLE and SLE and Controls

	Healthy donors (N=61)	APS patients (N=41)	P#	APS+SLE patients (N=31)	P#	SLE patients (N=54)	P#
CLINICAL PARAMETERS*							
Females/Males	43/18	33/8		27/4		50/4	
Age, (years)	39.02±9.86	48.53±11.58	n.s.	44.94±11.75	n.s.	36.48±11.80	n.s.
Anti-dsDNA	1.41±2.52	--		29.70±60.65	0.040	21.06±29.12	0.001
aCL IgG (GPL)	4.34±7.55	53.85±96.31	0.003	13.66±25.45	0.027	7.95±11.94	n.s.
aCL IgM (MPL)	8.79±6.23	28.78±51.17	0.020	18.72±44.55	0.009	10.70±47.96	n.s.
Anti-ß2GPI (SGU)	5.65±8.82	44.06±95.10	0.005	3.56±6.60	n.s.	2.44±4.99	n.s.
LA positivity (%)	0	29(71%)		15(48%)		13(24%)	
SLEDAI	0	--		2.10±3.82	0.021	1.56±2.19	0.001
Thrombosis (%)	0	28(68%)		28(90%)		2(4%)	
Obesity (%)	2(3%)	10(24%)		11(35%)		11(20%)	
Hypertension (%)	2(3%)	3(7%)		6(19%)		8(15%)	
Diabetes (%)	0	0		5(16%)		1(2%)	
Nephropathy (%)	0	0		7(23%)		15(28%)	
Increased CIMT (%)	3(5%)	13(32%)		10(32%)		11(20%)	
Corticosteroids (%)	0	3(7%)		22(71%)		29(54%)	
Antimalarials (%)	0	3(7%)		20(65%)		32(59%)	
Anticoagulants/antiplatelets(%)	0	33(80%)		26(84%)		31(57%)	
LABORATORY PARAMETERS*							
Total cholesterol (mg/dL)	190.08±34.95	191.65±35.89	n.s.	199.42±37.39	n.s.	180.25±30.10	0.010
Cholesterol HDL (mg/dL)	55.83±13.21	52.53±15.08	n.s.	50.80±15.35	n.s.	54.82±13.42	n.s.
Cholesterol LDL (mg/dL)	115.77±29.63	114.62±30.88	n.s.	125.00±30.24	n.s.	107.57±24.83	0.015
Triglycerides (mg/dL)	89.87±53.33	115.89±68.44	0.047	129.03±50.16	0.001	91.26±42.08	n.s.
C reactive protein (mg/dL)	2.34±5.41	6.11±10.83	0.046	5.72±9.14	0.001	2.47±3.53	0.019
Apolipoprotein A (g/L)	150.65±29.00	135.83±22.04	0.030	145.86±33.87	n.s.	146.80±27.31	n.s.
Apolipoprotein B (g/L)	78.55±20.22	86.08±19.23	n.s.	92.86±21.72	0.049	75.38±16.92	n.s.
C3 (mg/dL)	135.92±45.17	123.54±33.58	n.s.	125.13±46.69	n.s.	110.54±29.58	0.001
C4 (mg/dL)	26.94±11.82	23.06±7.89	n.s.	20.27±8.17	0.005	18.32±8.75	0.001

GPL indicates IgG phospholipid units; MPL, IgM phospholipid units; and SGU, standard IgG units.

*Except where otherwise indicated, values are the number of subjects and mean ± SD.

3.1 Blood samples

Plasma and serum samples, and purified monocytes (non-monocytes depleting kit, Miltenyi Biotech, Bergisch Galdbach, Germany) were obtained from peripheral venous blood samples as described elsewhere[26,27] (see supplementary online methods).

3.2 Flow cytometry analyses, Flow-cytomix and analysis of oxidative stress biomarkers in white blood cells and plasma.

See supplementary methods for details

3.3 B-Mode Ultrasound IMT Measurements and thrombosis assessment

B-mode ultrasound imaging for CIMT (carotid intima media thickness) measurements was performed as previously described[28,29] by using a Toshiba equipment (Aplio platform) equiped with 7-10 MHz broadband linear array transducers. For further details, see supplementary Methods.

3.4 Microarray analysis

Microarray studies were performed in an Agilent G4112F platform (Whole Human Genome Microarray 44k) using the One-Color gene expression system. These microarrays contain ~41,000 human genes and transcripts with one 60-mer oligonucleotide probe representing each sequence. See Supplementary Methods for further details. The raw microarray data were deposited in the Gene Expression Omnibus database of the National Center for Biotechnology Information, accession no. GSE50395.

3.5 Quantitative Real-time PCR

Changes of a number of transcripts highlighted by array results or related to the inflammatory/oxidative processes, were validated by quantitative

real-time RT-PCR using the LightCycler thermal cycler system (Roche Diagnostics, Indianapolis, USA), using GAPDH as housekeeping gene, as described elsewhere[26,27].

3.6 Western blot

Specific antibodies against IL6R, SLC25A27 (Abnova, Heidelberg, Germany), IFIT1 (Abcam, Cambridge, UK) and GPx8 (Santa Cruz, Madrid, Spain) were used to determine protein levels by Western blotting[26,27].

3.7 Purification of IgG and in vitro exposure of normal monocytes to aPL antibodies

IgG from the pooled sera of 7 patients with APS plus SLE (characterized by high titers of aCL antibodies, ie, >120 IgG phospholipid units) and from the pooled sera of 7 healthy subjects (as controls) was purified by protein G-Sepharose high-affinity chromatography (MAbTrap kit; Amersham Biosciences). Purified normal monocytes (1.5 $x10^6$ cells/mL) were incubated either with normal human serum (NHS)–IgG (500 μg/mL) or purified APS plus SLE patient (SAPS)-IgG (500 μg/mL) for 6 hours at 37°C.

3.8 Statistical analysis

All data were expressed as mean ± SD. Statistical analyses were performed with SSPS 15.0 (SPSS Inc., Chicago, IL, USA). Following normality and equality of variance tests, comparisons were made by paired Student's t test or alternatively by a non-parametric test (Mann–Whitney rank sum test). Correlations were assessed by Pearson product–moment correlation and association studies were performed through Chi-square test. The independent association between different variables in univariate analysis was determined by multivariate regression analysis. Differences were considered significant at $p<0.05$.

4. RESULTS

4.1 Microarray study: Functional categorization of genes differentially expressed among APS, APS plus SLE and SLE patients in the area of atherosclerosis, inflammation and CVD.

The number of genes differentially expressed in monocytes from patients vs controls was found significantly higher in APS plus SLE (1224) in relation to APS (518) and lupus patients (555). A comparative analysis allowed us to identify 1243 genes differentially expressed between APS and SLE, and 605 genes between APS plus SLE and SLE. Interestingly, only 220 genes were found differentially expressed in monocytes from APS patients compared with those of APS plus SLE, indicating a relatively small change between these two pathologies (Figure 1A).

Molecular network analysis showed that 20-30% of the total number of genes differentially expressed in the three pathologies was directly or indirectly related to atherosclerosis, inflammation and CVD. As a general feature, the main alterations were derived from the change in the expression of specific genes for each pathology, even although the number of genes differentially expressed was similar, and all of them belonged to the gene cluster of atherosclerosis, inflammation and CVD (Figure 1B-E, supplemental figures 1 and 2).

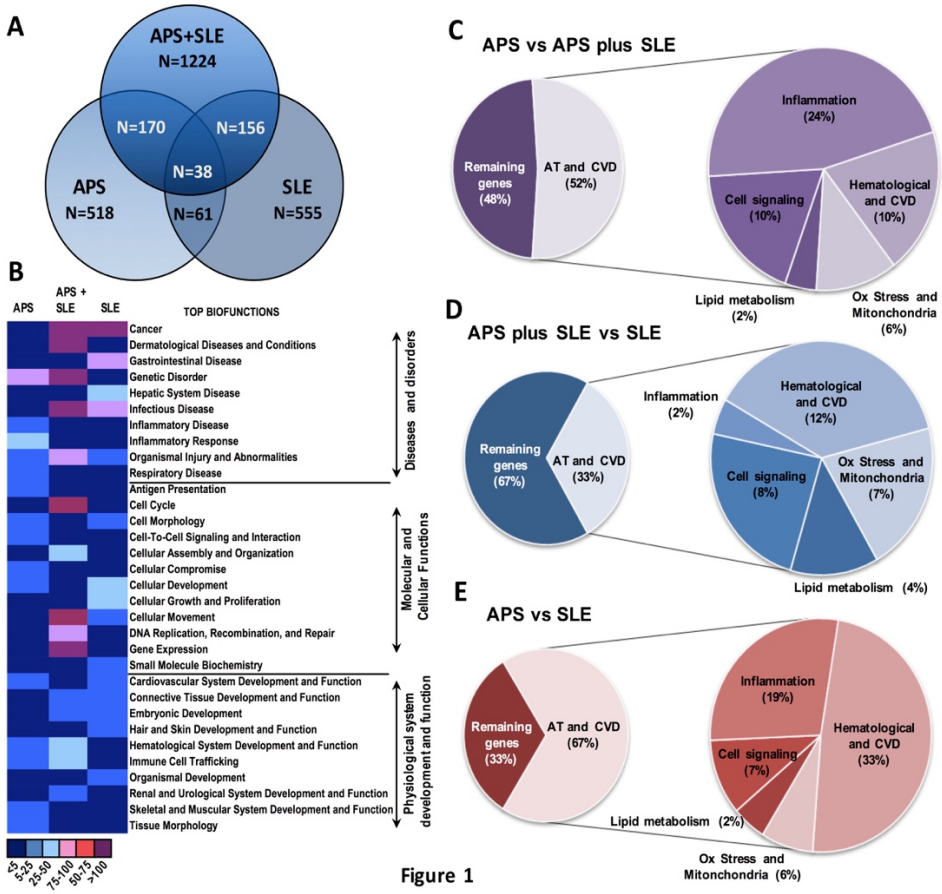

Figure 1

Figure 1. Overlapping and functional categorization of gene expression signatures among APS, APS plus SLE and SLE patients. (A) Venn diagram illustrating the overlap between the gene signatures that distinguish APS, APS plus SLE and SLE. Numbers in the diagram refer to number of genes. Those inside the circles refer to the number of genes differentially expressed in each respective disease vs. healthy donors. The numbers inside the crossing circles refer to the genes simultaneously expressed in two or the three diseases. B. Heat map of deviated functional categories in differentially expressed genes of APS, APS+SLE and SLE patients vs healthy donors. Each category (distributed by top biofunctions) is ranked by a range of colours (purple, pink, light blue, dark blue) showing the number of genes differentially expressed. (C-E). Diagrams showing the functional categorization of genes differentially expressed among diseases in the area of atherosclerosis and CVD. Differentially expressed genes were classified and used for computational analysis to identify potential functional pathways and networks using the Ingenuity Pathways Analysis Knowledge Base (Ingenuity Systems).

In relation to LES, monocytes from APS patients displayed differentially expressed genes involved in the biogenesis and function of mitochondria, including small molecule transporters (SLC25A27), regulators of

membrane polarization and potential (TP53TG5). Also some genes related to oxidative stress and antioxidant defense were found differentially expressed, with increased expression of GPx8, CYBA and PXDN. Many other genes related to atherosclerosis were found upregulated, including those codifying for vascular endothelial growth factor receptors (KDR, FLT4), blood coagulation and circulation (ITGA2, ELN, LPL), VEGF signaling [PI3 kinase signaling (PIK3R3, PIK3R5), small G-protein signaling (KRAS, SHC2), phospholipase A2 (PLA2G2E, PLA2G5) and protein phosphatases (PPP3R2)], cell adhesion molecules (CDH5), extracellular protease inhibitors (SERPINE1, PLG, TIMP3), transcription factors (ID3), chemokines (CXCL5), lipoprotein signaling and cholesterol metabolism (APOA4, CEL, HMGCS2, PRKAG2, APOF), and fatty acid metabolism (ACOXL, ACSM5, GK, GK5, GPD1, LPL).

In the inflammatory pathway, a number of genes were found differentially expressed including chemokines, interleukins and their receptors, interferons, growth factors, genes belonging to the TNF super family, IFN responsive genes, and genes regulators of the inflammatory response (Figures 2 and 3). The most relevant group of genes that mediate inflammatory signaling in cells included those related to the Wnt signaling pathway, the NFkappaB signaling pathway and the MAP kinase signaling pathway.

In addition to the interferon signature (represented by genes such as IFI27, IFI35, IFI44, IFI44L, IFI6, IFIT1, IFIT5, IFITM1, IFITM4P, etc), shared by APS plus SLE and SLE patients, differentially expressed atherosclerotic/inflammatory genes further included in APS plus SLE a significant number of those codifying for cytokines (IL6, IL11RA, IL16, IL22RA, IFNAR1) chemokines and their ligands/receptors (CCL13, CXCL17),

adhesion molecules (COL4A6, NRP1), and genes mediating inflammatory response (ITGA10, TOLLIP, NOX1, CYP26B1, LIFR, NLRP11, TRAF3, etc) (Figures 2 and 3).

Monocytes from APS and APS plus SLE patients shared a high number of altered genes, with the most relevant differences found in those codifying in APS for some atherogenic molecules such as lipoprotein a (LpA), a recognized independent risk factor for premature atherosclerotic coronary heart disease[29], and cytokine receptors such as IL22RA1, which is produced by PBMCs cultured in the presence of aPLs, suggesting its role in the pathogenesis of APS[30] (Figures 2 and 3).

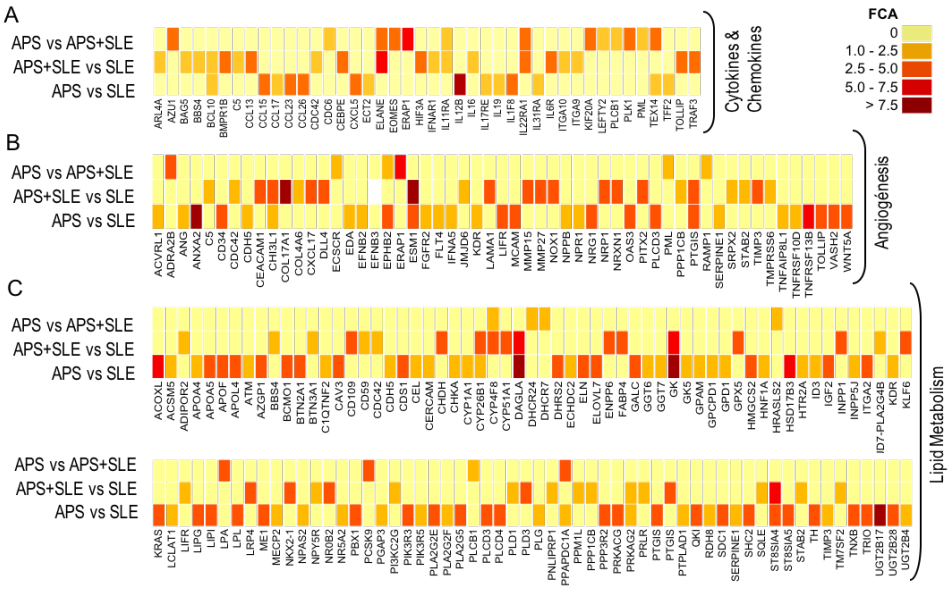

Figure 2. Functional categorization of genes differentially expressed between monocytes of APS, APS+SLE and SLE patients in the area of Atherosclerosis, Inflammation and Cardiovascular disease. (A-C) Heat maps depicting the expression microarray data for the genes differentially expressed between the three autoimmune conditions with P<0.05. FCA denotes fold change absolute.

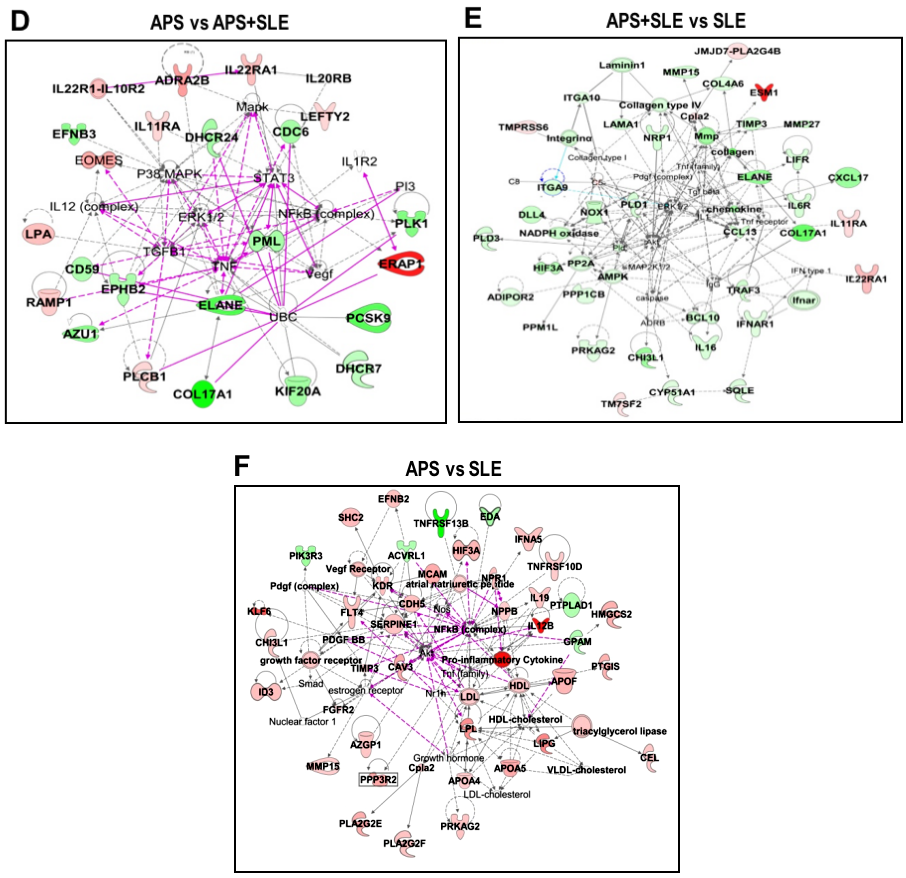

Figure 2. Functional categorization of genes differentially expressed between monocytes of APS, APS+SLE and SLE patients in the area of Atherosclerosis, Inflammation and Cardiovascular disease. (D-F) Gene networks showing inter-relationship between differentially expressed genes in the 3 disorders using Ingenuity Pathways Analysis software. Over-expressed genes are shown in red and under-expressed genes appear in green. Direct interactions appear in the network diagram as a solid line, whereas indirect interactions as a dashed line.

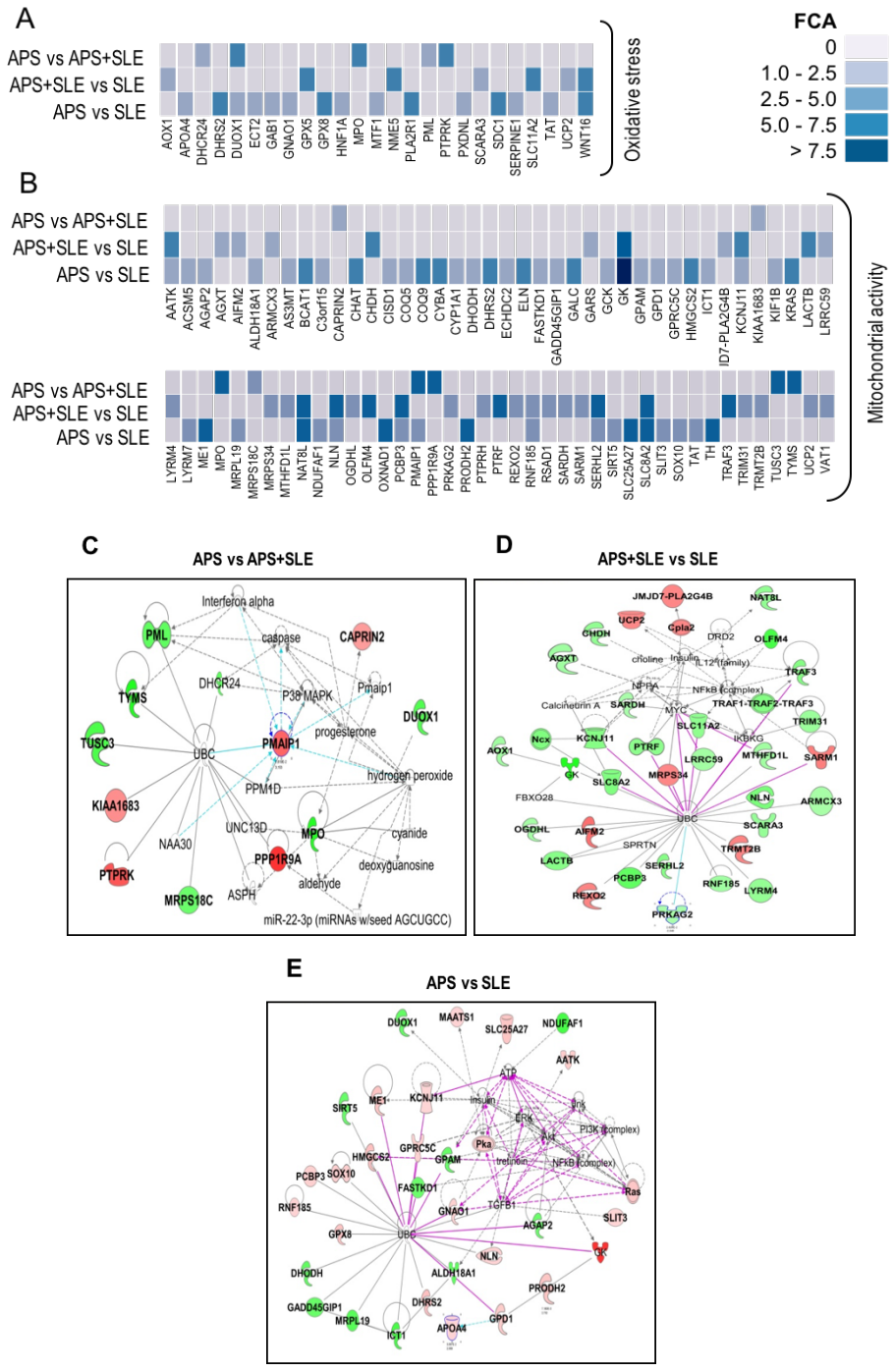

Figure 3. Functional categorization of genes differentially expressed between monocytes of APS and SLE patients in the area of oxidative stress and mitochondrial activity. (A,B) Heat maps depicting the expression microarray data for the genes differentially expressed between the three autoimmune conditions with P<0.05. FCA denotes fold change absolute. (C-E) Gene networks showing inter-relationship between differentially expressed genes in the 3 disorders using Ingenuity Pathways Analysis

software. Over-expressed genes are shown in red and under-expressed genes appear in green. Direct interactions appear in the network diagram as a solid line, whereas indirect interactions as a dashed line.

To further validate the data obtained we performed two set of analysis:

1. Measurement of protein production by monocytes (supplemental Figure 3), which suggested that the changed expression of some genes resulted in altered protein levels, thus providing evidence for a pathogenic role in the three studied diseases.

2. Monocytes from a number of patients of each pathology included in the study (five APS, seven APS+SLE and ten SLE, and ten controls) were evaluated again two years after the first blood sample collection, to analyze the stability of changes observed in the array. For this purpose, we performed RT-PCR of various genes found changed in the microarray and validated in the first study. Results (supplemental figure 4) demonstrate that gene expression in the second sample collection changed in the same way when they were first analyzed. Thus, our data support the theory that there is a specific Ath/CVD/inflammation signature characteristic of each disease, which remains stable along time.

4.2 Relationship between clinical and molecular markers of atherothrombosis and the presence of Antiphospholipid antibodies in APS plus SLE patients

Coexistence of APS and the presence of aPLs are recognized risk factors for sub-clinical atherosclerosis and CVD development in lupus. Thus we further searched for a link between clinical and molecular markers of atherosclerosis, inflammation and CVD, and the presence of aPLs.

Firstly, we found that, contrary to APS, APS plus SLE and SLE patients displayed a significant number of inflammatory deregulated markers (Table II). Yet, approximately a 35% of APS plus SLE patients showed increased pathologic CIMT, while in LES the frequency was 20%. Multivariate analysis showed that aCL titers independently predicted both the occurrence of thrombosis (standardized ß coefficient 0.463; P=0.001) and the presence of an increased CIMT (standardized ß coefficient 0.318; P=0.012), thus supporting an atherogenic/prothrombotic role for aCL antibodies of IgG isotype in APS plus SLE (SAPS) (Figure 4 A and B).

Plasma, cell surface and mRNA analyses of inflammatory and oxidative stress markers validated and complemented some of the results obtained in the array in APS plus SLE (Table II). We further found a significant correlation between IgG-aCL titers and monocytes mRNA levels of some well known inflammatory molecules including VEGF, IL-8, IL-1, and MCP-1, endothelial dysfunction markers (tPA), as well as antioxidant defense markers such as the glutathione peroxidase activity (Figure 4 C to H).

Moreover, in vitro treatment of monocytes with IgG-aCL significantly changed the expression of the genes involved in those processes, including some of those found most significantly altered in their expression in APS plus SLE by using the microarray approach vs healthy donors, such as CCL2, IL11RA, scl25a27, ARHGEF5, IFIT1 and PPARG (Figure 4 I).

Table II. Parameters related to inflammation and oxidative stress in APS, APS+SLE and SLE patients

	Healthy donors (N=61)	APS patients (N=41)	P#	APS+SLE patients (N=31)	P#	SLE patients (N=54)	P#
INFLAMMATORY PARAMETERS*							
Tissue Factor (%)	13.94±12.53	22.13±17.46	0.025	21.76±22.49	0.022	18.34±13.97	0.035
VEGF-A (pg/mL)	350.42±240.96	605.26±500.73	0.047	488.62±335.66	0.035	592.51±503.87	0.05
IFN-α (pg/mL)	126.33±207.81	87.60±145.71	n.s.	84.03±133.01	n.s.	198.47±303.46	n.s.
IFN-γ (pg/mL)	5.42±10.44	1.89±6.29	n.s.	0.27±0.75	n.s.	5.01±13.94	n.s.
IL-1ß (pg/mL)	12.98±21.26	13.45±18.82	n.s.	7.06±9.03	n.s.	33.09±45.51	n.s.
IL-2 (pg/mL)	26.97±38.44	36.81±82.31	n.s.	88.33±113.10	0.025	155.07±213.39	0.002
IL-6 (pg/mL)	0.08±0.12	0.03±0.10	n.s.	4.00±7.23	0.005	2.13±5.76	0.049
IL-8 (pg/mL)	7.51±22.24	26.68±52.76	0.045	27.51±44.13	0.001	22.11±49.85	0.023
IL-10 (pg/mL)	23.41±65.14	22.32±61.17	n.s.	0.31±0.81	n.s.	0.91±1.44	n.s.
IL-17 (pg/mL)	5.30±19.81	--		42.59±69.78	0.023	107.44±220.89	0.004
IL-23 (pg/mL)	5.03±26.15	--		19.43±36.60	0.043	93.23±207.82	0.007
MCP-1 (pg/mL)	494.96±159.56	610.41±269.95	0.027	1222.19±720.47	0.001	766.01±673.87	0.013
MIP1-α (pg/mL)	267.79±351.91	453.86±502.26	0.047	919.27±2777.75	n.s.	763.92±1756.36	n.s.
tPA (ng/mL)	1.94±0.82	2.64±1.41	0.015	3.79±1.61	0.001	3.89±3.01	0.011
OXIDATIVE STATUS*							
TAC (plasma; mM Trolox equiv)	150.98±71.85	104.11±44.92	0.001	126.42±36.98	n.s.	115.68±43.37	n.s.
Nitric oxide (plasma;µM)	22.49±13.81	15.39±8.17	0.008	18.88±10.15	n.s.	12.16±6.89	0.001
Nitrotyrosine (µM)	19.29±20.48	88.96±113.19	0.032	34.70±25.75	0.017	31.89±23.55	0.035
Manganese-SOD (U/min/mL/µg prot)	0.08±0.04	0.34±0.16	0.021	0.18±0.22	0.026	0.26±0.19	0.001
Catalase (nmol/min/mL/µg protein)	222.82±113.92	86.22±51.53	0.012	138.89±102.53	0.028	181.56±137.61	n.s.
GPx (nmol/min/mL/µg protein)	2.14±0.81	1.19±0.59	0.003	1.37±0.77	0.047	1.71±0.75	n.s.

* Values are mean ± SD

# P<0.05 vs healthy donors

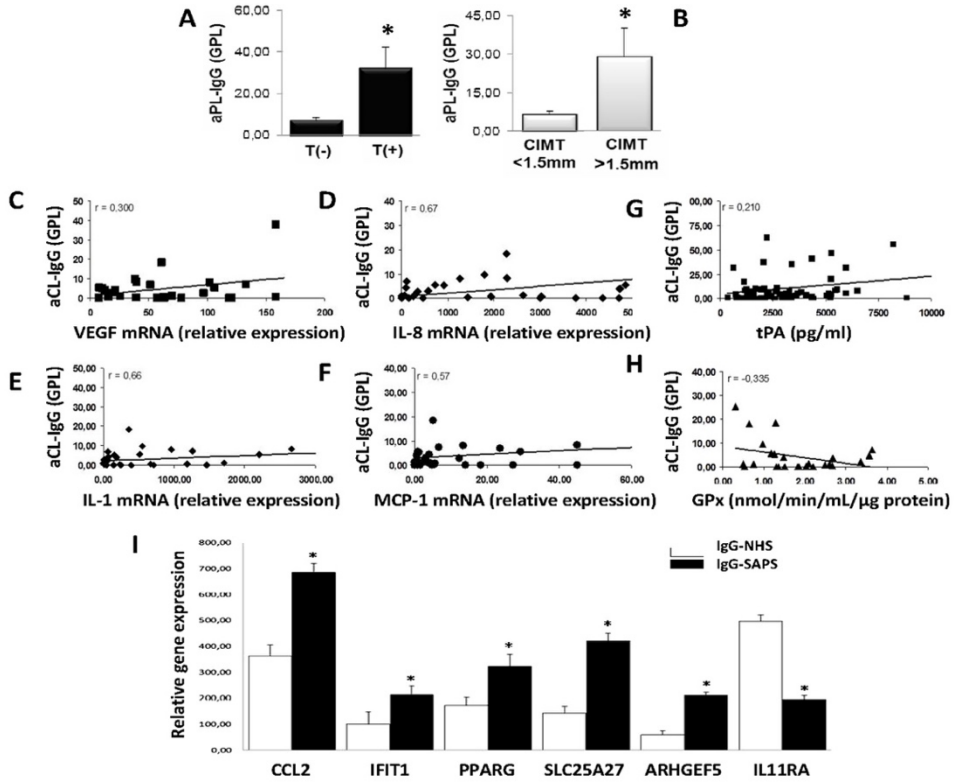

Figure 4. Association and correlation studies and in vitro effects of IgG-SAPS on the expression of the most significantly differentially expressed genes in APS+SLE in the area of atherosclerosis and CVD. (A-B) Relationship between IgG isotype anticardiolipin antibodies levels and the presence of an increased CIMT and (A) or the occurrence of thrombotic events (B). Asterisk (*) indicates significant differences either vs patients without increased CIMT or vs patients without thrombosis (P<0.05). (C-H) Correlation analyses between IgG isotype anticardiolipin antibodies levels and mRNA relative expression levels of VEGF, IL-8, IL-1, MCP-1 (C-F), plasma levels of tPA (G), and GPx activity (H). (I) Monocytes isolated from healthy donors were incubated for 6 hours with IgG-SAPS or IgG-NHS. Then, total RNA was extracted and RT-PCR of selected genes was performed as described in materials and methods. SAPS, Secondary APS or APS+SLE; NHS, Normal Human Serum. Values are means and SEM from four independent experiments. *Significant differences (at P<0.05) vs. monocytes treated with IgG-NHS.

5. DISCUSSION

This is the first study drawing simultaneously gene expression patterns of monocytes in patients with APS, APS plus SLE, and SLE. A number of studies have compared gene expression in peripheral blood mononuclear cells (PBMC) from patients with SLE vs healthy individuals or other autoimmune conditions[30-35] but only a few studies have focused on gene expression of cell sub-populations pertinent to SLE pathogenesis[36-39]. From those studies we could conclude that while array analysis of PBMC provide some useful information, the use of purified cell subsets identifies many more differentially expressed disease-specific genes. Such analyses provide substantial advantages in the search for diagnostic and prognostic biomarkers in autoimmune diseases. In the present study, the comparative gene profiling of three highly-related autoimmune disorders, identified multiple previously unreported genes differentiating them, thus enhancing our current understanding of these three diseases in aspects related to atherosclerosis and CVD.

APS, APS plus SLE, and SLE have been associated with accelerated atherosclerosis or other types of vasculopathies leading to increased cardio- and cerebrovascular disease risk. Traditional risk factors, as well as systemic inflammation including cytokines, chemokines, adipokines, proteases, autoantibodies, adhesion receptors, oxidative stress biomarkers, and a plethora of intracellular signalling molecules, have been implicated in the development of these vascular pathologies[40,1,2]. Yet, the characteristics of vasculopathies may significantly differ depending on the underlying disease.

Our microarray analysis revealed that there are a significant proportion of genes differentially expressed in these three pathologies that codify for proteins that may be involved in the development of atherosclerosis and CVD. Moreover, by integrating the gene profiling data from monocytes of APS, APS plus SLE, and SLE patients in the areas of CVD and oxidative stress using bioinformatics tools, disease-associated gene networks were identified. Pathways involved in cellular growth, thrombosis, inflammation (ERK1/2, AKT, PI3K, MAPK, p38MAPK) and immune reactions (STAT3, NFKB), were further recognized, implying an important role in the pathogenesis of the three autoimmune disorders.

Comparing among the three diseases, the high specificity found in the genes differentially expressed on each pathology would explain the differences and incidence of clinical manifestations. Thus, compared with SLE patients, a specific feature of APS monocytes was a significant number of differentially expressed genes involved in the mitochondria biogenesis and function, oxidative stress and antioxidant defense. These alterations have been previously reported to be directly related to thrombosis development in APS patients[8,9]. A strong interferon signature was found in monocytes from both SLE and APS plus SLE patients, thus corroborating previous studies that identified up regulation of type I IFN response genes in SLE[22,25,41], and explaining the concordance of some clinical features between both disorders. In addition, a number of genes were differentially expressed in APS plus SLE that might further account for the pro-atherothrombotic tendency of these patients. Finally, a close relationship between APS and APS plus SLE patients was found in the group of genes belonging to the cluster of atherothrombosis, probably as

a consequence of the aPL presence, which seems to drive this pathology in both autoimmune conditions[26,42,43].

A recent study demonstrated a direct relationship between the presence of both dysfunctional HDL and of carotid plaque in SLE patients and the alteration of monocyte atherosclerosis-related transcripts, such as CCL2, TNFα and PDGFRß[44]. Accordingly, our array also showed increased expression of PDGFRß in APS patients and PDGFRL in SLE patients. No changes were demonstrated in gene expression of CCL2 and TNFα, but some genes of the TNF family (TNFRSF17 in SLE and TNFRSF1A in APS) were found increased compared to control group.

Our study further suggested that APS plus SLE patients not only display a significantly different gene profile, but also appear to be at greater risk of developing certain pathological features, compared with those SLE patients who do not have aPL. Our results indicate that aPL-positive lupus patients have an increase in both, markers of early atherosclerosis development, and thrombotic events. A question that arises from that data is whether certain vascular manifestations, usually ascribed to lupus, are seen mainly or exclusively in those who have aPL.

Previous studies have shown that in APS plus SLE patients some clinical features that might be consistent with APS may not be caused by the presence of aPL[17]. Indeed, these manifestations may be caused by some other aspects of lupus. Nevertheless, our present data further indicate a relevant influence of aPL on SLE-specific thrombosis development, as demonstrated by the relationship between IgG-aPL titers in APS plus SLE patients and the occurrence of thrombotic events. That influence was further supported by the correlation between aCL-IgG titers and the levels

of monocytes mRNAs codifying for proteins directly related to thrombosis development.

Lupus patients have a 50-fold higher risk of developing atherosclerosis beyond the conventional risk factors[45]. In primary APS, IgG aPL has been demonstrated to be an independent predictor of increased IMT, a marker of atherosclerotic vascular disease[9,46]. We provide further evidence that aPL may be an additive risk for atherosclerosis in SLE that is not accounted for by the traditional risk factors (i.e. the significant association found between IgG-aPL titers and premature atherosclerosis). In support for this inference, in vitro studies demonstrated that a number of genes regulating all that processes are modulated by IgG-aPL.

In conclusion, our overall data suggest that gene expression profiling allows the segregation of APS, APS plus SLE and SLE, with specific signatures explaining the pro-atherosclerotic, pro-thrombotic and inflammatory changes in these highly related autoimmune diseases.

Many autoimmune disorders have common features of disease pathogenesis[47]. Although monocytes have been demonstrated to play a central role in atherosclerosis and CVD development, they are only one of the many players in the evolvement of these processes, which would further change depending on the presence of other traditional factors (age, obesity, diabetes, hypertension, etc) and the probable influence of the specific therapy in each group of patients, such as glucocorticoids, antimalarials, anti-platelets, etc. Thus, a complex interplay of different environmental, therapeutic and genetic factors can determine different clinical expression in those diseases. Nevertheless, the identification of relevant genes whose products regulate specific physiological pathways

might contribute to the development of targeted therapies for each autoimmune condition.

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Contributions

Contribution: S.M., P.R.-L. and C.P.-S. Developed the *in vivo* assays, performed the experiments and solved technical problems; M.A.A., M.J.C. and M.A.K. followed up with patients and contributed useful discussion and suggestions; A.R.A., N.B., and Ch.L-P. formed the hypothesis, directed and coordinated the project, designed the experiments, analyzed the data, and wrote the manuscript; E.C.-E. performed clinical analysis and contributed useful suggestions; Y.J.G. performed statistical analysis and discussed results.

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Competing interests. None.

Patient consent. Obtained.

Ethics approval This study was conducted with the approval of the Ethics Committee from the Reina Sofia Hospital from Cordoba-Spain.

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7. SUPPLEMENTARY INFORMATION

Blood samples

Peripheral venous blood samples were collected in sterile precooled tubes containing 0.129*M* sodium citrate (1/9 [volume/volume]; Becton Dickinson, Meylan,France). A selected volume of the samples was centrifuged at 4000 rpm. Plasma and serum were aliquoted and stored at -80°C until their analysis. Samples were used for the preparation of purified monocytes, lymphocytes and neutrophils, and for the estimation of lipid profile, inflammatory parameters, and the activity of oxidant and antioxidant enzymes. Laboratory markers of inflammation (C-reactive protein), clinical parameters (arterial or venous thrombosis, pregnancy morbidity) and traditional risk factors for atherosclerosis (obesity, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, apolipoproteins A and B), were established as part of routine patient management.

B-Mode Ultrasound IMT Measurements

All patients and controls underwent B-mode ultrasound imaging for Carotid Intima-media thickness (IMT) measurements. B-mode ultrasound imaging of the carotid arteries was performed as previously described (Ames PRJ et al., J Thromb Haemost 2009; Touboul PJ et al., Cerebrovasc Dis 2007) by using Toshiba equipment (Aplio platform) equipped with 7–10 MHz broadband linear array transducers.

Patients were examined in the supine position, with the head turned 45° contralateral to the side of scanning. Three carotid arterial segments were assessed: the common carotid (1 cm proximal to the bulb), the carotid bulb (between the dilatation and flow divider), and the internal carotid (1 cm distal to the flow divider). Of each segment, the near and

the far walls of the left and right carotid artery segments were imaged at 2 different angles. The maximum distance of the intima-media thickness, defined as "maximum IMT" was calculated for each view. Plaque was defined as a focal structure that encroached into the arterial lumen of at least 0,5 mm or 50% of the surrounding IMT value or demonstrated a thickness >1.5 mm as measured from the media-adventicia interface to the intima-lumen interface[38].

Flow cytometry analyses, analysis of oxidative stress biomarkers in purified monocytes and plasma.

Flow cytometry analysis

Flow cytometric analysis was performed in monocytes as previously described (Lopez-Pedrera et al., Arthritis Rheum 2006), using a FACScan (BD Biosciences, San Jose, California, USA) and monoclonal anti-human FITC-conjugated TF antibodies (clone TF9-6B4, American Diagnostica, Greenwich, Connecticut, USA), and anti-human FITC-conjugated PAR2 antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA).

FlowCytomix

CD40L, IFNα, IFNγ, IL-1, IL-2, IL-6, IL-8, IL-10, IL-17, IL-23, MCP-1, MIP-1α, TNFα, tPA, VEGF-A, and sP-selectin levels were quantified in sera using a cytofluorimetry-based ELISA system (Flowcytomix, Bender Medsystem GmbH, Austria). Two-colour cytometric analysis was performed using FACScalibur cytometer (Becton Dickinson Immunocytometry Systems (BDIS); San José, CA). Data were obtained and analysed using the FlowCytomix Pro software.

Determination of oxidative stress biomarkers in monocytes

Oxidative stress biomarkers were analysed in purified monocytes using a dual-laser FACSCalibur (Becton Dickinson, Mountain View, CA, USA). Test standardization and data acquisition analysis were performed using the CELL Quest software (Becton Dickinson). A forward and side scatter gate was used for the selection and analysis of the different cell subpopulations.

For the assessment of ROS generation, including superoxide anion and hydrogen peroxide, cells were incubated with 20.5 µM DCF-DA at 37°C for 30 min in the dark. For the joint detection of peroxides and peroxynitrites, cells were incubated with 5 µM DHR-123 at 37°C for 30 min in the dark. The change in mitochondrial membrane potential was monitored by incubating the cells with 5µM Rhodamine-123. The cells were washed, re-suspended in PBS, and then analysed on a dual-laser FACSCalibur. The JC-1 Mitoscreen assay (BD Biosciences) was used (final concentration 2 µM) to assess $\Delta\psi_m$ according to manufacturer's instructions.

Determination of plasma and cell oxidative stress biomarkers

The NO stable end products nitrite plus nitrate, were measured in plasma using a commercial kit (Total Nitric Oxide Assay Kit, Thermo Scientific, Rockford, IL, USA). Serum total antioxidant capacity (TAC) was measured by quantitative colorimetric determination, using TAC Assay Kit (BioVision, Mountain View, CA, USA). Nitrotyrosine, as a marker of nitrative stress, was measured in monocytes extracts with a competitive enzyme immunoassay (Cell Biolabs, San Diego, CA). Mitochondrial SOD activity (Mn-SOD), Catalase (CAT) activity and Glutathione peroxidase (GPx)

activity were assayed in cell lysates using specific kits (Cayman Chemical Company, MI, USA) according to manufacturer's instructions.

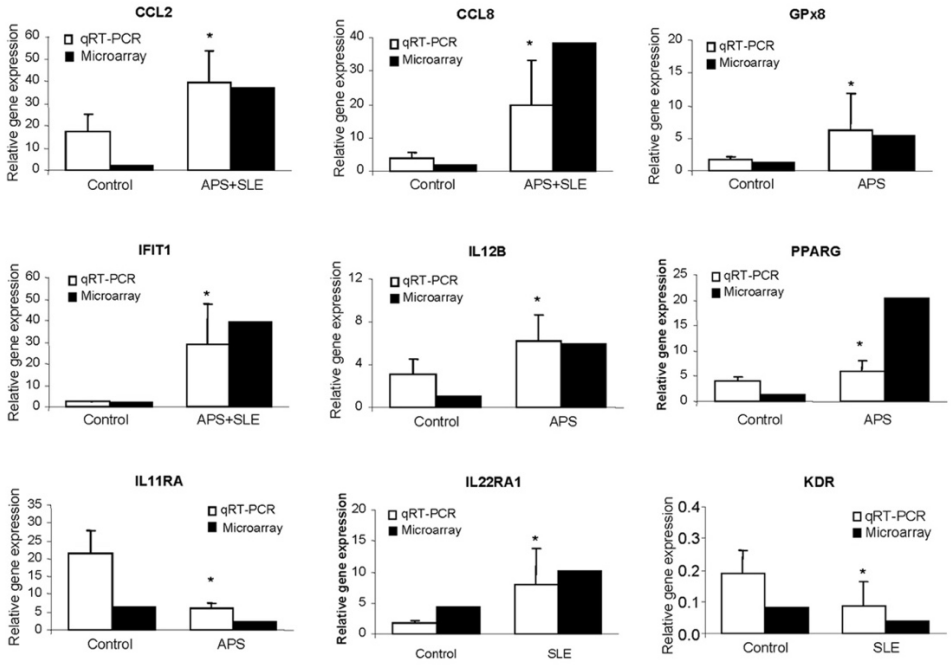
Microarray analysis

Total RNA was extracted from monocytes of APS, SLE and APS+SLE patients and healthy donors using TRIzol reagent (Invitrogen). RNA quality control was performed in a 2100 Bioanalyzer (Agilent Technologies). In all cases, the RNA integrity number was >7. Complementary RNAs were prepared for hybridization in an Agilent G4112F platform (Whole Human Genome Microarray 44k) using the One-Color gene expression system (Agilent Technologies). These microarrays contain ~41,000 human genes and transcripts with one 60-mer oligonucleotide probe representing each sequence.

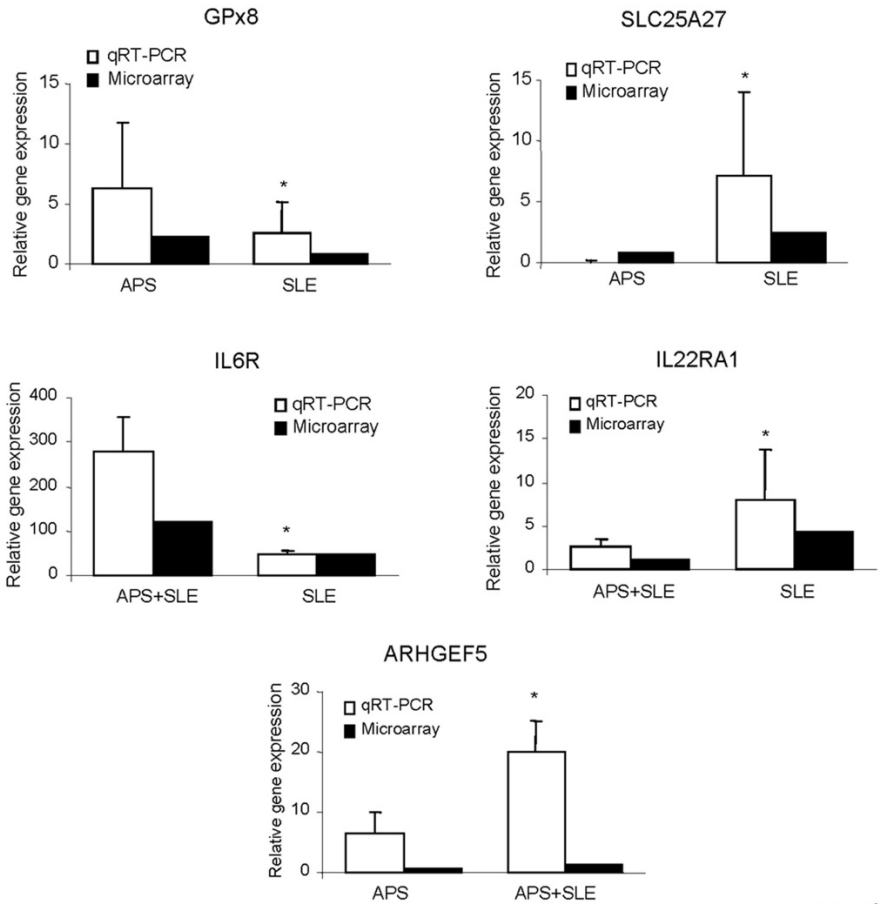
Microarray data were extracted with Feature Extraction Software version 10.7 (Agilent Technologies), and data analysis was carried out using GeneSpring GX version 10.0 (Agilent Technologies). Student's paired *t*-test with Benjamini and Hochberg multiple testing corrections was used to determine the statistical significance of the difference in gene expression between groups. *P* values less than 0.05 were considered significant. An additional cutoff threshold of 2-fold change in gene expression (either up-regulation or down-regulation) was used to define a gene as being differentially regulated. Differentially expressed genes were classified and used for computational analysis to identify potential functional pathways and networks using the Ingenuity Pathways Analysis Knowledge Base (Ingenuity Systems). The functional analysis identified those biologic functions that were most significant for the data set. Fisher's exact test was used to calculate a *P* value determining the probability that each

biologic function assigned to the genes based on the data was due to chance alone.

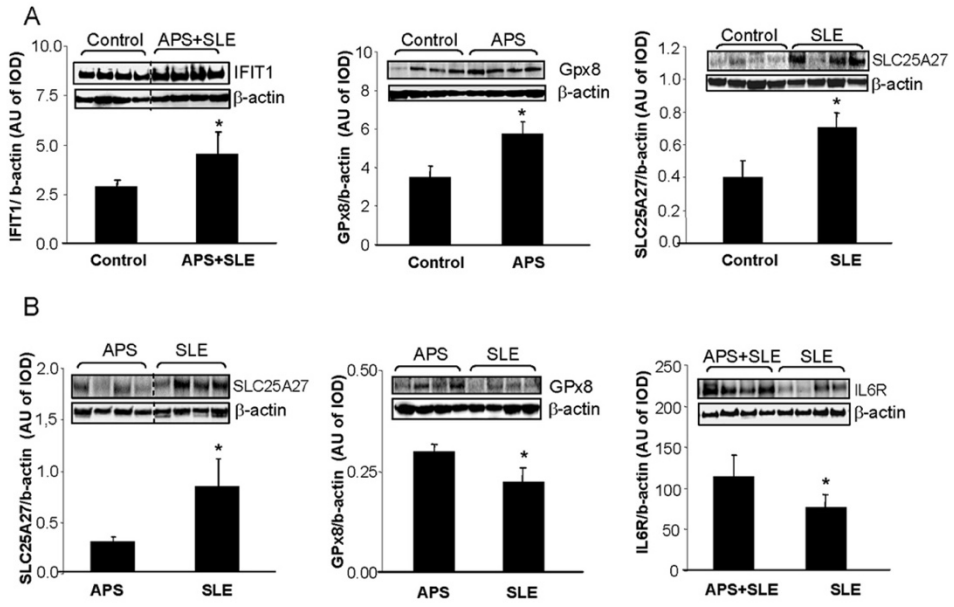
Supplemental figures



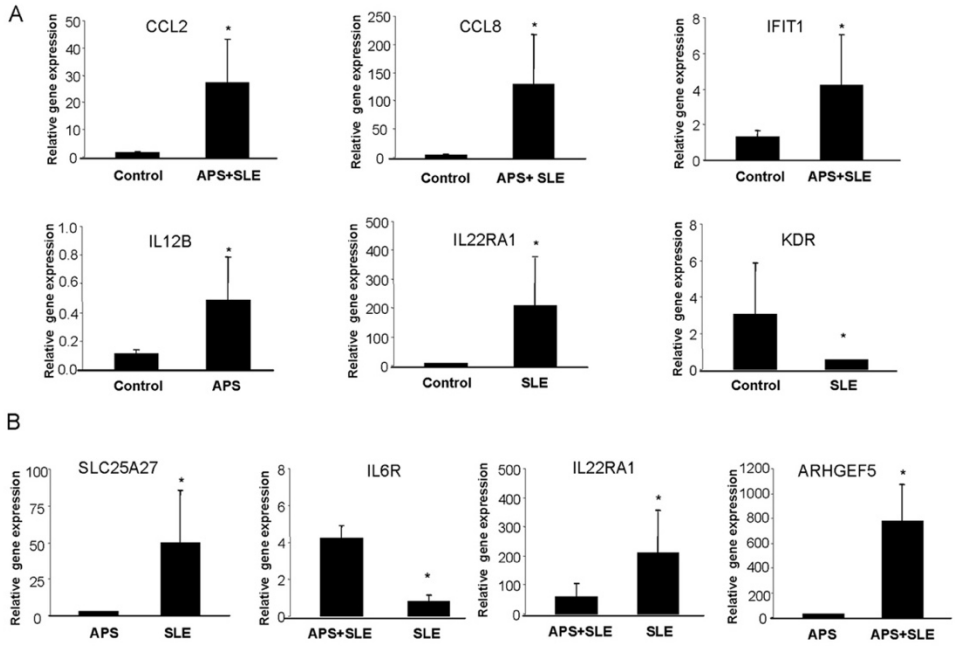
Supplemental Figure 1. Microarrays results validation by qRT-PCR of differentially expressed genes between patients and healthy donors. Quantitative real-time RT-PCR was performed on selected genes from patients and healthy donors under the basis of their degree of statistical significance, magnitude of differential gene expression, and possible biologic relevance to the diseases. PCR assays (empty bars) were consistent with the array results (solid bars). *P<0.05 vs healthy donors.



Supplemental Figure 2. Microarrays results validation by qRT-PCR of differentially expressed genes among the three autoimmune conditions. Quantitative real-time RT-PCR was performed on selected differentially expressed genes from APS, APS+SLE and SLE patients. PCR assays (empty bars) were consistent with the array results (solid bars). *P<0.05 vs the comparative disease.



Supplemental Figure 3. Microarray results validation by protein expression of differentially expressed genes among the patients and controls (A) and the three autoinmune disorders (B). Western blot analyses were performed on selected proteins from controls (n=10), APS (n=8), SLE (n= 7) and APS+SLE patients (n=8). Lanes were run on the same gel but were non-contiguous (IFIT1 and SLC25A27). *p<0.05 vs controls or the comparative disease.



Supplemental Figure 4. Microarray data validation through qRT-PCR of differentially expressed genes among the controls and patients (A) and the three autoinmune disorders (B). Quantitative real time PCR was performed on selected differentially expressed genes from patients and controls 2 years after the first sample collection: control (n=10), APS (n=5), SLE (n=10) and APS+SLE (n=7).*p<0.05 vs controls and the comparative disease.

CAPÍTULO III

SCIENTIFIC REPORTS

'Atherothrombosis-associated microRNAs in Antiphospholipid syndrome and Systemic Lupus Erythematosus patients'

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Atherothrombosis-associated microRNAs in Antiphospholipid syndrome and Systemic Lupus Erythematosus patients

JOURNAL: Scientific Reports (Sci. Rep.)

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1. ABSTRACT

MicroRNAs (miRNAs) markedly affect the immune system, and have a relevant role in CVD and autoimmune diseases. Yet, no study has analyzed their involvement in atherothrombosis related to APS and SLE patients. This study intended to: 1) identify and characterize miRNAs linked to CVD in APS and SLE; 2) assess the effects of specific autoantibodies. Six miRNAs, involved in atherothrombosis development, were quantified in purified leukocytes from 23 APS and 64 SLE patients, and 56 healthy donors. Levels of miRNAs in neutrophils were lower in APS and SLE than in healthy donors. Gene and protein expression of miRNA biogenesis-related molecules were also reduced. Accordingly, more than 75% of identified miRNAs by miRNA profiling were underexpressed. In monocytes, miR124a and -125a were low, while miR-146a and miR-155 appeared elevated. Altered miRNAs' expression was linked to autoimmunity, thrombosis, early atherosclerosis, and oxidative stress in both pathologies. In vitro treatment of neutrophils, monocytes, and endothelial cells with aPL-IgG or anti-dsDNA-IgG antibodies deregulated miRNAs expression, and decreased miRNA biogenesis-related proteins. Monocyte transfections with pre-miR-124a and/or -125a caused reduction in atherothrombosis-related target molecules. In conclusion, miRNA biogenesis, significantly altered in neutrophils of APS and SLE patients, is associated to their atherothrombotic status, further modulated by specific autoantibodies.

2. INTRODUCTION

Accumulating evidence shows that humoral autoimmunity might play a relevant role in cardiovascular disease (CVD). Some autoantibodies, present in patients with antiphospholipid syndrome (APS) and systemic lupus erythematosus (SLE), possibly represent emerging cardiovascular (CV) risk factors. Thus, previous studies have demonstrated that antiphospholipid antibodies (aPL) provoke a pro-atherothrombotic state through the induced expression of both prothrombotic and proinflammatory molecules, as well as through the induction of oxidative stress and mitochondrial dysfunction in monocytes and neutrophils[1,2,3]. Furthermore, studies have shown that endothelial cells (ECs) expressed significantly higher amounts of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) when incubated with aPL antibodies and ß2GP1 in vitro[4,5]. Similarly, the incubation of ECs with antibodies reacting with ß2GP1 has been shown to induce EC activation with upregulation of tissue factor (TF)[6], adhesion molecules, IL-6 production and alteration in prostaglandin metabolism[5].

On the other hand, the immunologic hallmarks of lupus are autoantibodies against nuclear proteins and anti-double-stranded DNA, so that anti-dsDNA titres correlate with disease activity and are associated with specific tissue damage[7,8]. In addition, anti-dsDNA antibodies titres are linked to the expression of inflammatory mediators (in plasma and immune cells) that characterize that autoimmune condition [9,10]. Moreover, in vitro treatment of ECs with anti-DNA autoantibodies has been shown to upregulate IL-1, IL-6, IL-8, transforming growth factor beta, nitric oxide synthase, and adhesion molecules expression, thus providing

evidence that anti-dsDNA could play an important pathogenic role in inducing inflammatory injury of vascular endothelium in SLE [11,12,13].

Pathophysiological mechanisms connecting atherosclerosis and CVD with APS and SLE have been greatly broadened with the application of genomic technologies, which have allowed explaining how these alterations might be associated to each autoimmune disease[14,15,16]. One emerging and important mechanism controlling gene expression is epigenetics, which controls gene packaging and expression independent of alterations in the DNA sequence. Epigenetics, which comprises DNA methylation, histone modifications, and microRNA (miRNA) activity, is providing new directions linking genomics and environmental factors[17].

MiRNAs are small noncoding RNAs of approximately 19-25 nucleotides in length[18] originated as pri- and pre-miRNAs, and processed by different ribonucleases such as Drosha and Dicer. miRNAs are ubiquitously expressed in a wide range of species and negatively regulate gene expression at the post-transcriptional level by targeting specific mRNAs for degradation or suppressing mRNA translation[18,19]. The whole human genome is estimated to encode 2588 mature miRNAs (miRbase v21, June 2014), which are predicted to target one third of human genes[20].

A number of works have analyzed the expression profile of miRNAs in peripheral blood cells, biological fluids, and tissues of patients with SLE. These works have shown that differential expression of multiple miRNAs seems to contribute to SLE pathogenesis, by regulating the type I interferon pathway, inflammatory cytokine expression, DNA methylation in T cells and local tissue inflammation (i.e. miR-15, miR-21, miR-31, miR-125a, miR142, miR-146a, miR-155, and miR-181, among others)[21].

Moreover, the expression of deregulated miRNAs in SLE patients influences some parameters of the disease activity and severity[20,22]. In APS, miRNAs from miR-17-92 cluster were identified as potential modulators of the expression of TF, the main inductor of thrombosis in APS patients[23].

Some studies have highlighted the role of miRNAs in processes such as oxidative stress and CVD, including atherosclerosis[24]. However, no study has identified and characterized miRNAs associated with CV and atherothrombotic risks observed in APS and SLE.

Thus, we undertake this work: 1) to identify and to characterize miRNAs related to the pathogenesis of CVD in APS and SLE patients. 2) to assess the effects of specific autoantibodies in the regulation of those epigenetic processes.

3. METHODS

3.1 Patients

Eighty-seven patients, 23 with primary APS, and 64 with SLE, as well as 56 healthy donors were included in the study (during a period of 48 months). All experimental protocols were approved by the ethics committee of the Reina Sofia Hospital in Cordoba and written informed consent was obtained. All methods were carried out in accordance with the approved guidelines. Subjects were selected among the patients with stable disease for more than six months, without infections, abortions, thrombosis, or changes in their treatment protocol. None of the healthy controls had a history of autoimmune disease, bleeding disorders, thrombosis, or pregnancy loss. The characteristics of patients and controls are shown in Table I.

Table I. Clinical and Laboratory parameters of the Antiphospholipid Syndrome, Systemic Lupus Erythematosus patients and the Controls

	Healthy donors (N=56)	APS patients (N=23)	P#	SLE patients (N=64)	P#
CLINICAL PARAMETERS*					
Females/Males	36/20	16/7		56/8	
Age, (years)	36.44±9.19	44.90±15.		41.61±12.21	n.s.
Anti-dsDNA positivity (%)	0	0	n.s.	18 (28%)	0.001
aCL IgG positivity (%)	0	5(22%)	--	9(14%)	
aCL IgM positivity (%)	0	3(13%)		6(9%)	
Anti-ß2GPI positivity (%)	0	9(39%)		5(8%)	
aCL IgG (GPL)	2.86±4.32	21.52±32.56		9.45±17.99	0.015
aCL IgM (MPL)	6.05±6.13	8.83±10.72	0.023	18.24±55.62	n.s.
Anti-ß2GPI (SGU)	4.07±7.17	45.84±94.37	n.s.	3.95±8.26	n.s.
LA positivity (%)	0	7/10 ((70%)	0.007	12/24 (50%)	
SLEDAI	0	--		1.69±2.53	0.001
Thrombosis (%)	0	19 (83%)		26(40%)	
Arterial Thrombosis (%)	0	16 (84%)		21(81%)	
Venous Thrombosis (%)	0	5 (26%)		9(35%)	
Fetal loss (%)	0	8 (35%)		7 (11%)	
Obesity (%)	1 (2%)	6 (26%)		15 (23%)	
Hypertension (%)	0	4 (17%)		10 (15%)	
Diabetes (%)	0	0		3 (5%)	
Smoking (%)	7 (13%)	4 (17%)		22 (34%)	
Hyperlipidemia (%)	3 (5%)	3 (13%)		10 (15%)	
Nephropathy (%)	0	0		17 (26%)	
Increased CIMT (%)	2 (4%)	9 (39%)		13 (20%)	
Corticosteroids (%)	0	2 (9%)		46 (71%)	
Antimalarials (%)	0	5 (22%)		43 (66%)	
Anticoagulants/antiplatelets (%)	0	18 (78%)		44 (68%)	
LABORATORY PARAMETERS*					
Total cholesterol (mg/dL)	191.18±33.99	192.73±39.37	n.s.	185.83±33.60	n.s.
Cholesterol HDL (mg/dL)	55.74±13.15	44.86±10.83	0.001	51.78±14.17	n.s.
Cholesterol LDL (mg/dL)	117.70±29.01	122.57±35.78	n.s.	113.95±27.41	n.s.
Triglycerides (mg/dL)	86.89±48.36	143.59±138.95	n.s.	102.72±44.47	n.s.
C reactive protein (mg/dL)	1.95±5.40	3.40±4.73	n.s.	3.06±3.61	n.s.
Apolipoprotein A (g/L)	146.69±28.24	135.55±17.16	n.s.	146.87±30.81	n.s.
Apolipoprotein B (g/L)	77.26±16.69	84.70±24.25	n.s.	77.52±19.15	n.s
C3 (mg/dL)	124.27±44.59	123.46±29.19	n.s.	103.89±28.22	0.010
C4 (mg/dL)	24.01±9.23	21.74±6.62	n.s.	17.65±7.47	0.001

GPL indicates IgG phospholipid units; MPL, IgM phospholipid units; and SGU, standard IgG units.

*Except otherwise indicated, values are number of subjects and mean ± SD.

We further studied 20 patients with thrombosis but without aPL (14 non-pregnant women and 6 men, mean age 45 (range: 24-63 years), including patients with objectively verified thrombotic events: 8 deep venous thrombosis and 12 thrombosis in intra-cerebral vessels).

3.2 B-Mode Ultrasound IMT Measurements

All patients and controls underwent B-mode ultrasound imaging for CIMT (carotid intimate media thickness) measurements as previously described[9,16].

3.3 Blood samples

The collection of peripheral venous blood samples for obtaining plasma, serum, and for purifying monocytes (non-monocytes depleting kit, Miltenyi Biotech, Bergisch Galdbach, Germany), lymphocytes, and neutrophils (dextran sedimentation) was performed as described elsewhere[9, 16].

3.4 Flow cytometry analyses, and analysis of oxidative stress biomarkers in purified leukocytes and plasma

Flow cytometric analysis was performed in monocytes as previously described[9] using a FACScan (BD Biosciences, San Jose, CA,). The BD Cytofix/Cytoperm fixation/permeabilization kit was used to analyse the intracellular expression of some cytokines (i.e. IL-6, IL-8, MCP-1), according to manufacturer's instructions.

CD40L, IFNα, IFNγ, IL-1, IL-2, IL-6, IL-8, IL-10, IL-17, IL-23, MCP-1, MIP-1α, TNFα, tPA, VEGF-A, and sP-selectin levels were quantified in sera using a cytofluorimetry-based ELISA system (Flowcytomix, Bender Medsystem GmbH, Austria)[3].

Oxidative stress biomarkers were analysed in purified leukocytes using a dual-laser FACSCalibur (Becton Dickinson, Mountain View, CA) as previously described[3,9].

Mitochondrial SOD activity (Mn-SOD), Catalase (CAT) activity and Glutathione peroxidase (GPx) activity were assayed in cell lysates using specific kits (Cayman Chemical Company, ciudad, MI).

3.5 Western Blotting

Dicer, p38 MAPK, Erk, STAT-3, GAPDH and actin protein levels were determined by Western blotting[3,9], using specific antibodies (Abcam and Santa Cruz Biotechnology).

3.6 In silico studies

Databases and algorithms of miRNA target prediction were used for the search of miRNAs targeting prothrombotic and proinflammatory mediators. We used TargetScan (release 5.1: http://www.targetscan.org), which provides the prediction results computed by the TargetScanS algorithm[25], PicTar (http://pictar.mdc-berlin.de)[26] , and miRanda (http://www.microrna.org/microrna/home.do)[27].

3.7 RNA isolation and qRT-PCR for mRNA and microRNA expression

Total RNA from lymphocytes, monocytes, and neutrophils was extracted using TRI Reagent (Sigma, St Louis, MO) following manufacturer's recommendations. MiRNA biogenesis modulators such as Dicer, Drosha, Argonaute-1, Argonaute-2, Exportin-5, and other inflammatory molecules were quantified as previously described[9,16].

For quantification of mature miRNA levels, trizol purified RNA was used. cDNA was synthesized from 200 ng RNA using individual miRNA-specific

RT primers contained in the TaqMan® MicroRNA Reverse Transcription Kit. Each cDNA was amplified using the TaqMan® MicroRNA assays together with TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Life Technologies, Madrid, Spain). The $2^{-\Delta Ct}$ method was used to calculate the relative abundance of miRNAs compared with U6 snRNA expression.

3.8 NanoString nCounter assay

For miRNA expression data generation, the NanoString human v2 array, which contains 800 miRNA probes, was used. Pools with RNA purified of neutrophils from 5 SLE patients and from 5 healthy donors were performed. A total of 100 ng RNA input was used per sample and conditions were set according to the manufacturer's recommended protocol (NanoString Technologies; Seattle, WA). Data were normalized by the geometric mean of all targets using the nSolver software.

3.9 Purification of IgG and in vitro exposure of white blood cells and endothelial cells to aPL antibodies

IgG from the pooled sera of 7 patients with APS (characterized by high titres of anti-cardiolipin –aCL- and anti-ß2GPI antibodies) was purified by protein G-Sepharose high-affinity chromatography (MAbTrap kit; Amersham Biosciences) following the manufacturer's recommendations. Briefly, MAbTrap Kit contains a HiTrap™ column prepacked with Protein G Sepharose™, a Type III Fc receptor that binds to the Fc region of all IgG fractions present in serum or plasma by a chromatographic method. Anti-ß2GPI and IgG-aCL activities of purified IgG were confirmed by enzyme-linked immunosorbent assays (QUANTA Lite® ß2GPI-IgG and QUANTA Lite® ACA IgG III kits, Inova Diagnostics; San Diego, CA, USA).

For *in vitro* studies, monocytes and neutrophils purified from healthy donors were incubated either with 10 µg/ml LPS, synthetic human IgG (40 µg/mL) (Jackson InmunoResearch Laboratories, Inc, Newmarket, Suffolk, UK) or purified APS patients-IgG (40 µg/mL) for 6 hours at 37°C.

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Group Ltd (Basel, Switzerland) and cultured in Endothelial Basal Medium (EBM, Lonza, Walkersville, MD USA) supplemented with 10% fetal bovine serum (FBS, Lonza), 0.1% human epidermal growth factor (hEGF, Lonza), 0.1% hydrocortisone (Lonza), 0.1% Gentamicin-Amphotericin-B (GA-1000, Lonza), 0.4% bovine brain extract (BBE, Lonza), and 1% Zell Shield (Minerva Biolabs, GmbH, Berlin, Germany) at 37ºC and 5% CO_2. Confluent cell monolayers were treated for 6 hours at 37°C with aPL-IgG and anti-dsDNA antibodies, or with LPS or synthetic human IgG, as described above. All the experiments were performed on passage 4.

3.10 Purification of anti-dsDNA IgG from SLE patients and in vitro culture of white blood cells and endothelial cells

Anti-dsDNA IgG antibodies from the pooled sera of 7 SLE patients (characterised by high titres of anti-dsDNA) were purified using a commercial kit (Quanta Lite-INOVA Diagnostics, San Diego, CA) through the ELISA-elution assay method. Monocytes, neutrophils, and ECs, were then incubated with 10 µg/ml LPS, synthetic human IgG (40 µg/ml) or purified Anti-dsDNA IgG from the pooled sera of 5 SLE patients (40 µg/ml) for 6 h at 37°C.

3.11 Cell transfection

Monocytes purified from healthy donors were plated 24 hours before transfection in 6-well plates with complete medium without antibiotics

(Opti-MEM, Life Technologies, Madrid, Spain). Cells were transfected with 100 nmol/L miRNA mimic (Life Technologies, Madrid, Spain) for miR-124a, miR-125a -either separately or in conjunction-, and a non-specific control (scrambled) by using siPORT^{TM} NeoFX^{TM} transfection agent (Life Technologies, Madrid, Spain). After 48 hours, cells were activated with 10 µg/mL LPS (Sigma-Aldrich, Madrid, Spain) for 6h, and potential targets were analyzed. Data were expressed as changes relative to the values of the cells transfected with scrambled control.

3.12 Statistical analysis

All data were expressed as mean ± SD. Statistical analyses were performed with SSPS 17.0 (SPSS Inc., Chicago, IL, USA). Following normality and equality of variance tests, comparisons were made by paired Student's t test or alternatively by a non-parametric test (Mann–Whitney rank sum test). Correlations were assessed by Spearman rank correlation test and association studies were performed through Chi-square test. Differences were considered significant at $P<0.05$.

4. RESULTS

4.1 Bioinformatic prediction of miRNAs having specific targets related to thrombosis and inflammation and quantification in leukocyte subsets

In silico studies were performed in order to determine the possible regulation of prothrombotic and proinflammatory molecules by miRNAS. We used the web-tool miRo (http://ferrolab.dmi.unict.it/miro)[28], to allow the simultaneous visualization from the results of three different restrictive algorithms Targetscan (http://www.targetscan.org), PicTar (http://pictar.bio.nyu.edu), and miRanda (http://microrna.sanger.ac.uk).

Indeed, our research focused on those miRNAs that were predicted to repress those proinflammatory molecules by the three algorithms. The most relevant miRNAs identified following this method were miR-124a-3p, miR-125a-5p, miR-125b-5p, miR-146a-5p, miR-155-5p, and miR-222-3p. Potential mRNA targets involved in processes such as atherothrombosis, immune response, oxidative stress and intracellular signaling were identified through the use of QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). We further developed a network that integrates the interaction miRNA-mRNA target (Figure 1).

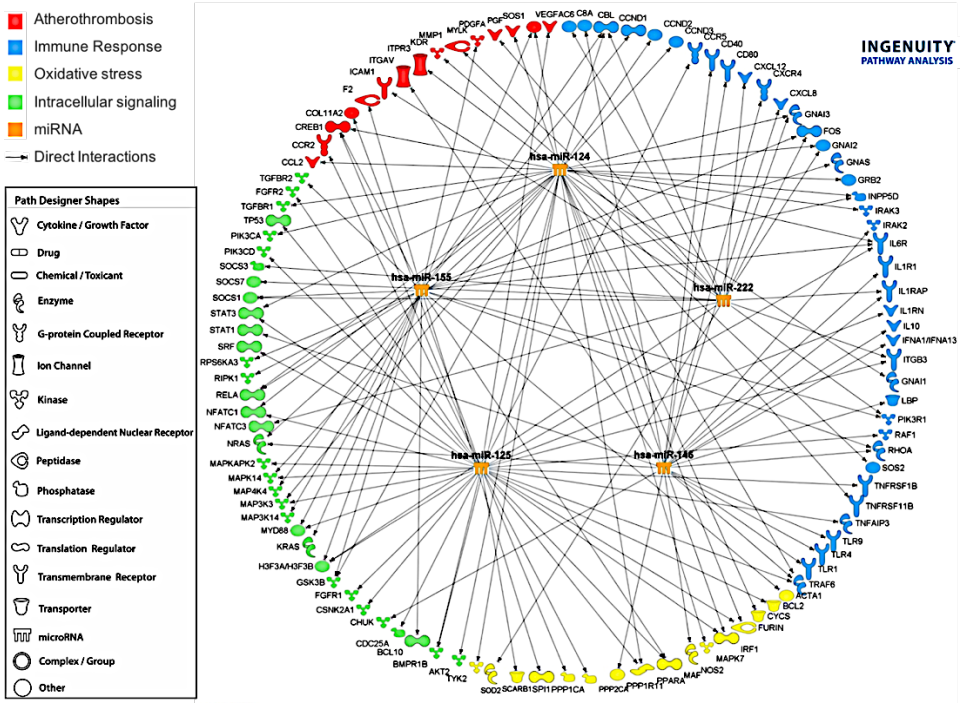

Figure 1. Interaction Network of miRNAs and mRNA target involved in cardiovascular disease (atherothrombosis, immune response, oxidative stress and intracellular signaling). By using the tool microRNA Target Filter of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity), the software generated a network including the selected miRNAs and their mRNA targets, filtered by connective tissue disorders. Only targets experimentally observed and predicted with high confidence are shown and related by direct interactions with their specific miRNA regulators.

Analyses performed on both APS and SLE patients showed that the expression levels of all the selected miRNAs in neutrophils were found significantly decreased in relation to the control group (Figure 2 A). Mir124a and miR-125a were also found reduced in monocytes from APS and SLE patients, while miR-146a and miR-155 appeared significantly increased (Figure 2 B). No significant changes in the expression of miRNAs were found in lymphocytes from APS or SLE patients (data not shown).

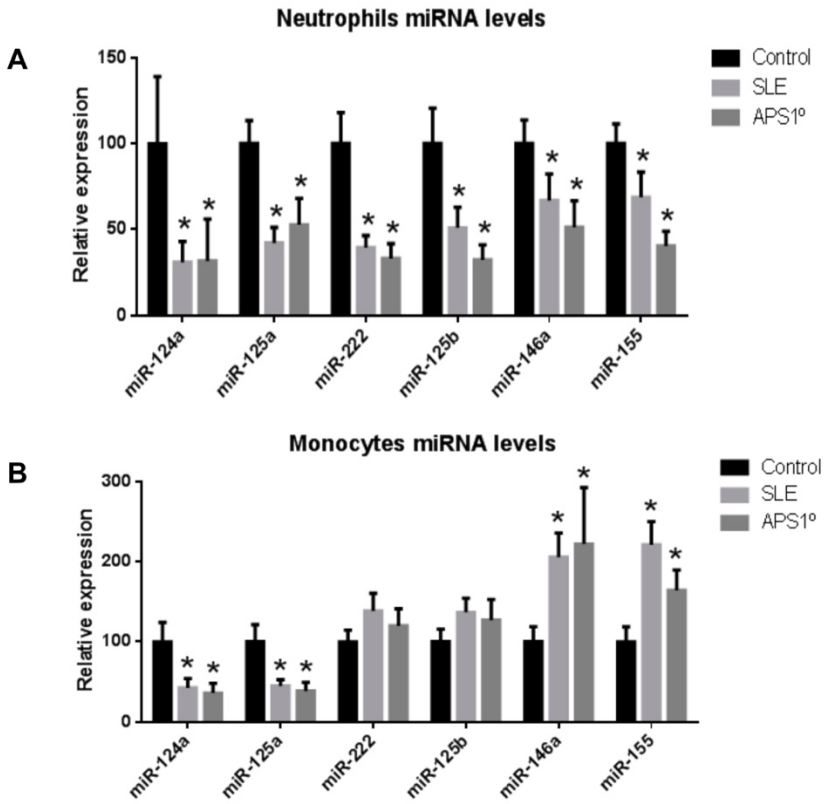

Figure 2. Expression levels of the selected miRNAs in monocytes and neutrophils from APS and SLE patients. miRNAs levels were measured in all the subjects included in the study (23 APS, 64 SLE, and 56 healthy donors) on isolated neutrophils (A) or monocytes (B) by qRT-PCR and normalized with U6 snRNA. Differences were analyzed by means of Student's t test. Statistical significance was taken as $p<0.05$.

4.2 Potential influence of therapy on miRNA modulation in APS and SLE patients

Numerous studies reported that miRNAs' expression can be modulated by effect of specific therapies[29]. To assess this aspect in our cohort of patients, we classified them in three groups that could discriminate the treatments received, including primary APS patients, SLE patients negative for aPL, and SLE patients positive for aPL. Then, we distributed them on different groups of treatment, on which we performed statistical analyses.

On primary APS patients (n=23), we identified 17 patients that had been treated only with oral anti-platelets/anticoagulants (A/A), 3 treated with A/A plus antimalarials (hydroxychloroquine, HCQ), two patients that received A/A+HCQ+prednisone, and 1 patient with no drug treatment (Supplementary Table IV). Comparison between patients treated with A/A vs those treated with A/A+HCQ showed no significant differences on miRNA expression (data not shown).

On SLE patients positive for aPL (n=23), we found 12 patients that received combined treatment with A/A, HCQ, and prednisone; 5 patients treated with A/A plus prednisone; 3 patients taking A/A + HCQ + Prednisone + Immunosuppressive drugs; 2 patients treated with A/A; and one patient treated with A/A+HCQ (Supplementary table V). Statistical analyses between groups of patients receiving AA+HCQ+prednisone vs patients treated with AA+prednisone demonstrated no significant differences on miRNA expression (data not shown).

Finally, on SLE patients negative for aPL (n=41), we verified a high homogeneity in the treatments administered, so that almost all the patients received A/A, HCQ, and prednisone. We could only distinguish a number of patients treated with immunosuppressive drugs (n=10) from those not taking that drug (n=31) (Supplementary Table VI). Comparative analyses between those groups demonstrated a significant increase in the expression of miR146 and miR155 in monocytes of SLE patients that received immunosuppressive therapy (Azathioprine or Mycophenolate mofetil) in relation to the ones non-treated with those drugs (data not shown).

4.3 Biomarkers of miRNA biogenesis are underexpressed in neutrophils from APS and SLE

The mRNA expression levels of miRNA biogenesis proteins (Dicer, drosha, Ago-1, Ago-2 and Exportin 5) were found significantly reduced (except for drosha in SLE) in neutrophils from APS and SLE patients (Figure 3A). No changes were found in monocytes (data not shown). Analysis of Dicer expression by Western blot showed that the expression of this protein appeared reduced by 68 % and 34 % in neutrophils from SLE and APS patients, respectively (Figures 3B-E), indicating a potential defect in miRNA biogenesis pathway in neutrophils of these patients.

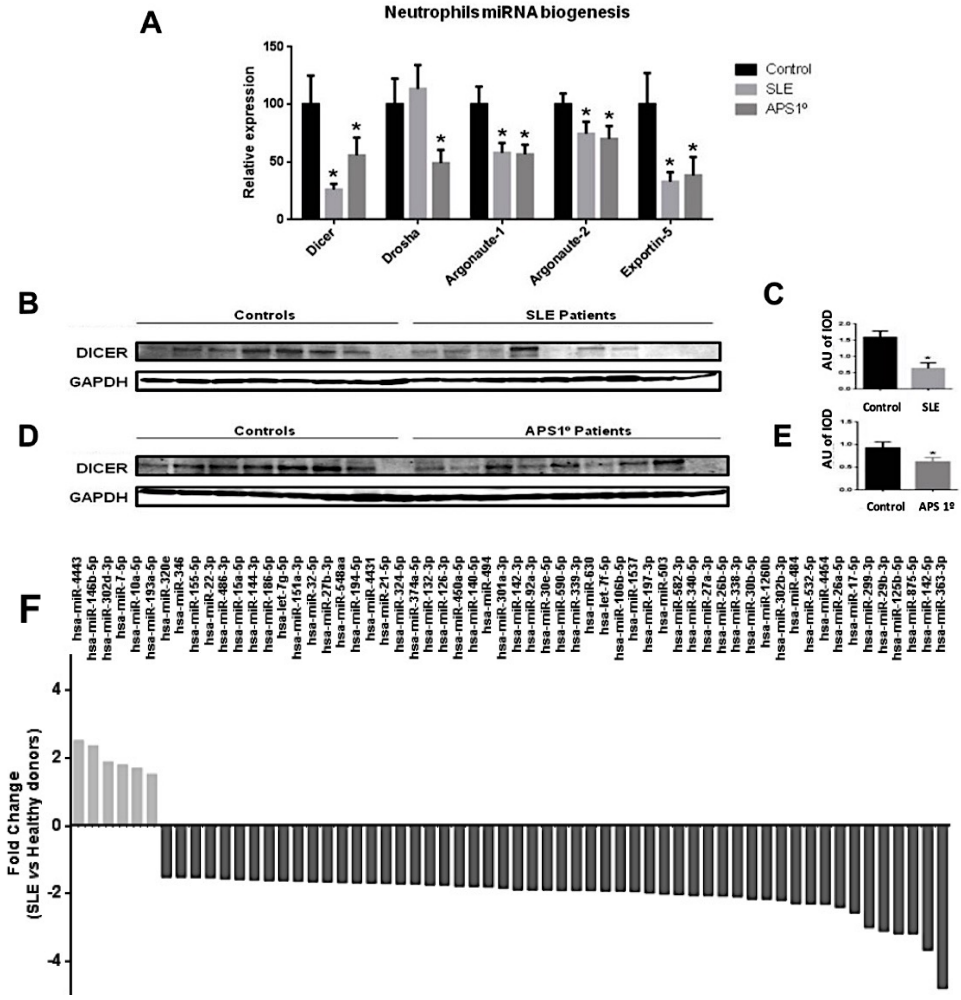

Figure 3. Biomarkers of miRNA-biogenesis in neutrophils from APS and SLE patients and comparison of the miRNA expression profiles in neutrophils from SLE patients' vs healthy donors. (A) Relative mRNA expression levels of miRNA biogenesis proteins, including *Dicer*, Drosha, Ago1, Ago2 and Exportin-5, evaluated in isolated neutrophils from all the subjects included in the study (23 APS, 64 SLE, and 56 healthy donors) by qRT-PCR. (B) Representative Western blots of protein Dicer detection in neutrophils from 9 SLE patients and 8 healthy donors performed in triplicate as described in the Materials and methods section. (C) The bar graphs show mean protein expression levels ± SEM – expressed as arbitrary units (AU) of integrated optical density (IOD)- from all the SLE patients and controls included in the study. (D) Representative Western blots of protein Dicer detection in neutrophils from 9 primary APS patients and 8 healthy donors. (E) The bar graphs show mean protein expression levels ± SEM from all the primary APS patients and the controls included in the study. Asterisks (*) indicate significant differences (at $P<0.05$) *vs* healthy donors. (F) NanoString assays were performed as described in Materials and Methods. miRNAs showing a fold change of at least 1.5 between patients and healthy donors are represented.

4.4 NanoString nCounter assay showed that most miRNAs are significantly underexpressed in SLE neutrophils

The nCounter profiling identified the expression of 163 miRNAs in neutrophils from SLE patients and healthy donors. Of these, 129 (79,1%) were underexpressed and 34 (20,9%) were overexpressed in neutrophils from SLE patients. Among them, 60 miRNAs showed at least 1,5-fold change between patients and healthy donors (Figure 3F). Those results suggest that the alteration in the expression of miRNA biogenesis proteins might lead to reduced miRNAs expression in neutrophils from SLE patients.

4.5 Non-autoimmune patients with previous thrombotic events show differential miRNAs alteration than APS and SLE patients

In order to assess whether the altered expression of the miRNA evaluated was linked to the thrombophilic state of APS and SLE patients, or was a sign of the immune activation, an additional control group, including 20 patients with thrombosis but without aPL was evaluated.

The expression levels of the selected miRNAs in neutrophils were not altered in relation to healthy donors, except for miR125b, which appeared significantly increased (Supplementary Figure S3A). In monocytes, miR125a, miR-222, miR-125b, miR146a, and miR155 were found significantly reduced (Supplementary Figure S3B).

4.6 Positivity for aPL in SLE patients influences the expression of miRNAs associated to thrombosis development

SLE patients were sub-grouped according to aPL positivity, and statistical analyses were performed to identify the changes occurred in the selected miRNAs –linked to CVD- evaluated in this study. In monocytes, a significant increase in two miRNAs (miR-146a and miR-155) was demonstrated in aPL-positive SLE in relation to the aPL-negative SLE patients (Figure 4A). aPL-(+) SLE patients showed a significant reduction in the expression of four out of the six miRNAs in neutrophils in relation to aPL-(-) SLE patients (miR-124a, miR-125a, miR-222, and miR155) (Figure 4B). Accordingly, a number of miRNA-biogenesis genes were also found reduced in those patients (Figure 4C).

In addition, aPL-positivity in SLE patients was linked to the development of thrombosis (around a 70% of SLE patients showing high titres of aPL had suffered at least one thrombotic episode), while a reduced number of aPL-(-) SLE patients had suffered thrombotic events (Figure 4D) (see supplementary tables V and VI for further details). In accordance with that results, proinflammatory profile also differed among aPL(-) and aPL(+)-SLE patients, so that while in aPL(-)-SLE patients cytokines such as IFNγ, IL-23, IL-1ß and TNFα were more prevalent, in aPL-(+) SLE patients an increased expression of tPA, PAR-2 and TF was noticed (Figure 4E and 4F).

We further analysed differences in miRNAs expression in SLE patients that had suffered previous thrombotic events and those without thrombosis in relation to healthy donors (Supplementary Figure S5) SLE patients T(+) showed significantly reduced levels of all the miRNAs evaluated in neutrophils in comparison with healthy donors, as well as reduced

expression of miR124a and miR125a, and increased expression of miR-155 in monocytes. Proteins related to miRNA biogenesis were found also significantly reduced in neutrophils.

In SLE patients T(-) we found significantly reduced levels of three of the six miRNAs evaluated in neutrophils, while in monocytes we identified reduced expression of miR124a and miR125a, as well as elevated expression of miR146a and miR155. Proteins related to miRNA biogenesis were, like in SLE patients T(+), significantly reduced in neutrophils.

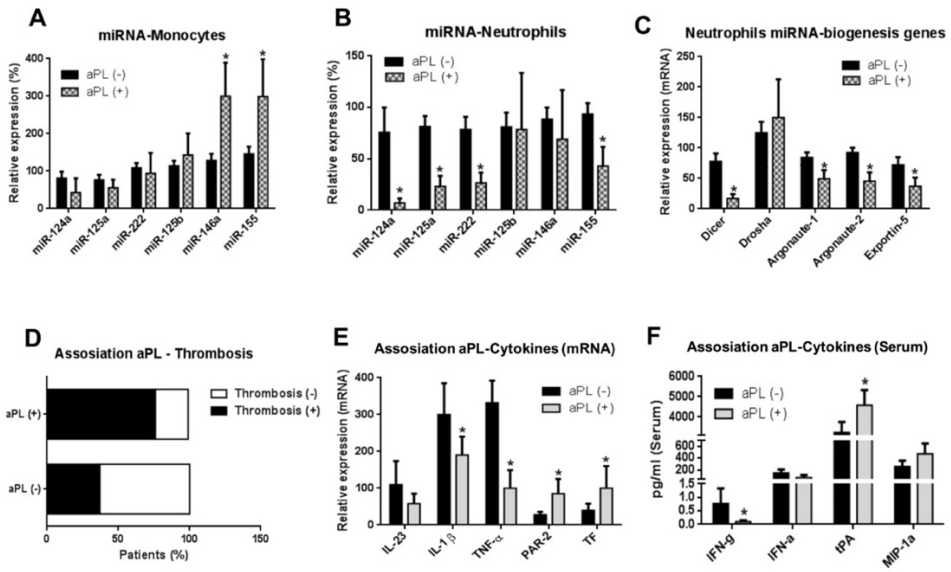

Figure 4. Expression of miRNAs, miRNA-biogenesis proteins, and inflammatory mediators in SLE patients positive for aPL versus SLE patients negative for aPL. SLE patients were sub-grouped according to aPL positivity, and statistical analyses were performed to identify the changes occurred in the selected miRNAs (Student's test). miRNA levels were measured in monocytes (A) and neutrophils (B) isolated from SLE patients with or without aPL by qRT-PCR, and normalized with U6 snRNA. Statistical significance was taken as $p<0.05$. (C) Relative mRNA expression levels of miRNA biogenesis proteins, including Dicer, Drosha, Ago1, Ago2 and Exportin-5. (D) Relationship between the positivity for aPL and occurrence of thrombotic events in SLE patients. (E) Relationship between the positivity for aPL and the gene expression of inflammatory parameters in monocytes from SLE patients. (F) Relationship between the positivity for aPL and the expression of inflammatory parameters in the plasma from SLE patients.

4.7 Prothrombotic, Inflammatory, and oxidative stress parameters are deregulated in APS and SLE patients

As reported in previous studies[3,6,16], in our set of patients, APS monocytes showed increased cell surface expression of TF and PAR2 (Supplementary Table SI). APS patients also displayed increased plasma levels of IL-8, MCP-1, and tPA. SLE patients demonstrated increased levels of monocyte TF and PAR2, as well as augmented plasma levels of IL-6, IL-8, IL-17, IL-23, MCP-1, and t-PA.

Peroxide/peroxinitrites production was notably increased in monocytes and neutrophils of APS and SLE patients. In line with this, the percentage of cells with altered ΔΨm was found significantly increased in monocytes and neutrophils. A prominent increase in the activity of monocyte mitochondrial SOD was found in APS and SLE patients compared with healthy donors. Yet, the activities of CAT and GPx were notably reduced in both monocytes and neutrophils from APS patients, as well as in neutrophils from SLE patients.

4.8 Correlation and association studies

Correlation and association studies in SLE patients showed that the expression levels of the analyzed miRNAs in neutrophils were linked with some parameters related to autoimmunity, such as the presence of elevated autoantibody titers (i.e. anti-dsDNA over 50 and ACA-IgG over 25 GPL), and with plasma and cellular levels of different proinflammatory and proatherothrombotic proteins (Supplementary Table SII). Correlation analyses with markers related to oxidative stress further showed significant negative correlations with peroxide levels and with the percentage of cells with altered ΔΨm, as well as positive correlations with

CAT activity in neutrophils of SLE patients. Some of these correlations were also found among various miRNAs in monocytes of SLE patients.

Association studies showed that the occurrence of a thrombotic event was related to low expression levels of 4 of the 6 evaluated miRNAs in neutrophils as well as to both, the reduced levels of miR-125a and the increased expression of miR-155, in monocytes from SLE patients (Supplementary Figure S1A). The reduced expression of the 6 selected miRNAs in neutrophils and the altered expression of miR-146a and miR-155 in monocytes were also found associated with a pathological increase in the CIMT in these patients (Supplementary Figure S1B).

As in the case of SLE patients, correlation studies in APS patients showed that the expression levels of some miRNAs differentially expressed in neutrophils and monocytes, significantly correlated with parameters related to autoimmunity, as well as with different proteins related to thrombosis, inflammation, and oxidative stress (Supplementary Table SIII).

We also found in APS patients an association between the occurrence of thrombotic events and the altered levels of a number of miRNAs in neutrophils and monocytes (Supplementary Figure S2A). Levels of some of the miRNAs differentially expressed in monocytes and neutrophils from APS patients were further associated with a pathological increase in the CIMT (Supplementary Figure S2B).

Furthermore, association studies showed that the occurrence of thrombotic events and a pathological increase of CIMT were also associated with low levels of proteins related to miRNA biogenesis in both

SLE and APS patients (Supplementary Figures S1C and D, and 2 C and D, respectively).

4.9 Anti-phospholipid-IgG antibodies and anti-dsDNA-IgG modulate both the expression of selected miRNAs and the expression of Dicer

The expression of all miRNAs analysed was significantly reduced in neutrophils treated with either aPL-IgG or anti-dsDNA-IgG compared to those treated with synthetic human IgG (Figures 5A and 6A). Both autoantibodies also caused a significant decrease in the expression of Dicer in this cell type (Figures 5B and 5C and 6B and 6C). In monocytes, treatment with aPL-IgG or anti-dsDNA-IgG promoted a significant reduction in the levels of miR-124a, as well as increased expression of miR-146a and miR-155 (Figures 5D and 6D). Levels of Dicer remained unchanged (Figures 5E and 5F, and 6E and 6F). Likewise, in ECs both IgG-aPL and anti-dsDNA antibodies promoted a significant inhibition in the expression of miRNAs evaluated (Figures 5G and 6G), as well as a reduced expression of the biogenesis protein Dicer at both, mRNA and protein levels (Figures 5H and 5I, and 6H and 6I). A trend to a reduction in other biogenesis proteins was further observed (Supplementary Figure S4A). Accordingly, both autoantibodies induced upregulation of MCP-1, TF, and VCAM-1, and downregulation of eNOS, relevant markers of endothelial dysfunction, and potential targets of the miRNAs evaluated (Supplementary Figure S4B).

The administration of a positive control for inflammatory pathways and immune activation, such as LPS[30], further promoted significant changes in the miRNAs and biogenesis proteins evaluated, although that changes

were occasionally divergent from those induced by both autoantibodies, thus suggesting a differential modulation of cellular activation.

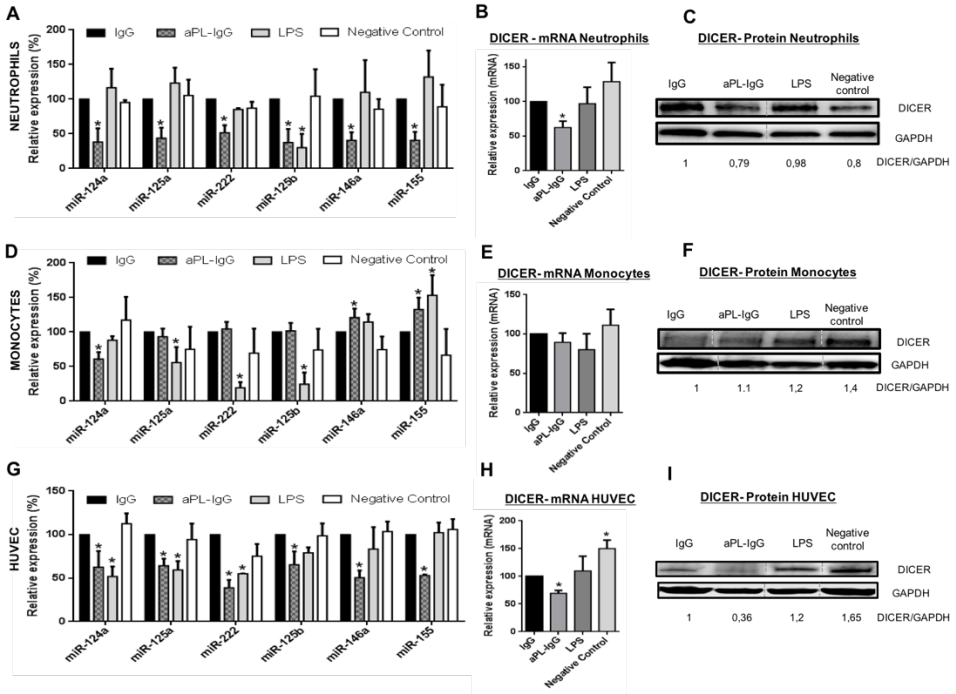

Figure 5. *In vitro* effects of aPL-IgG antibodies on both, the expression of selected microRNAs, and the expression of Dicer. Monocytes and neutrophils isolated from healthy donors, as well as primary human umbilical vein endothelial cells (ECs) were treated in vitro with anti-phospholipid IgG isotype antibodies (aPL-IgG) purified from APS patients' serum, or with LPS as positive control, or cell culture medium as negative control, or LPS as positive control, or culture medium as negative control, or synthetic human IgG. (A, D and G) Relative expression levels of selected miRNAs in neutrophils, monocytes, and ECs, respectively. Values are the means and SEM of 4 independent experiments. Significant differences (*P<0.05) *vs* neutrophils or monocytes or ECs treated with synthetic IgG. (B, E and H) Relative expression levels of *Dicer* mRNA in neutrophils, monocytes, and ECs treated with aPL-IgG, LPS, culture medium, or with synthetic IgG of 4 independent experiments. Significant differences (*P<0.05) *vs* cells treated with synthetic IgG. (C, F and I) Representative Western blotting results of 4 independent experiments showing Dicer expression after the treatment indicated in neutrophils, monocytes, and ECs. Lanes were run on the same gel under the same experimental conditions but were non-contiguous. Cropping lines are used in the figure. Full-length blots are presented in Supplementary Figure S6A.

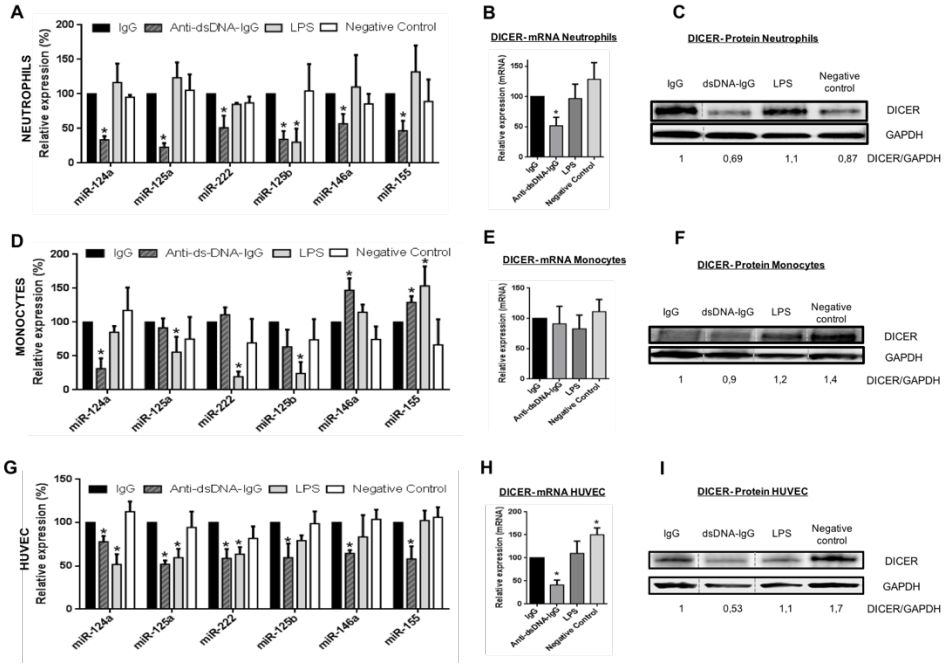

Figure 6. *In vitro* effects of anti-dsDNA-IgG antibodies on both, the expression of selected microRNAs, and the expression of Dicer. Monocytes and neutrophils isolated from healthy donors, as well as primary human umbilical vein endothelial cells (ECs) were treated *in vitro* with anti-dsDNA-IgG antibodies purified from SLE patients' serum, or LPS as positive control, or culture medium as negative control, or with synthetic human IgG. (A, D and G) Relative expression levels of selected miRNAs in neutrophils, monocytes, and ECs, respectively. Values are the means and SEM of 4 independent experiments. Significant differences (*P<0.05) *vs* neutrophils or monocytes or ECs treated with synthetic IgG. (B, E and H) Relative expression levels of *Dicer* mRNA in neutrophils, monocytes, and ECs treated with anti-ds-DNA-IgG, LPS, culture medium, or with synthetic IgG of 4 independent experiments. Significant differences (*P<0.05) *vs* cells treated with synthetic IgG. (C, F and I) Representative Western blotting results of 4 independent experiments showing Dicer expression after the treatment indicated in neutrophils, monocytes, and ECs. Lanes were run on the same gel under the same experimental conditions but were non-contiguous. Cropping lines are used in the figure. Full-length blots are presented in Supplementary Figure S6A.

4.10 Transfection with miR-124a and/or miR-125a mimics promoted decreased expression of inflammatory markers and their intracellular pathways

Monocyte transfections with miR-124a and miR-125a mimics, either separately or simultaneously, caused a reduction in the expression of target molecules related to the atherothrombotic process in APS and SLE, such as MCP-1, IL-6, IL6R, IL-8, ERK, STAT-3, p38 MAPK and peroxides (Figure 7A to 7F). The simultaneous transfection with both pre-miRNAs did not potentiate the inhibitory effect caused by each miRNA mimic administered separately, but increased the global number of molecules targeted.

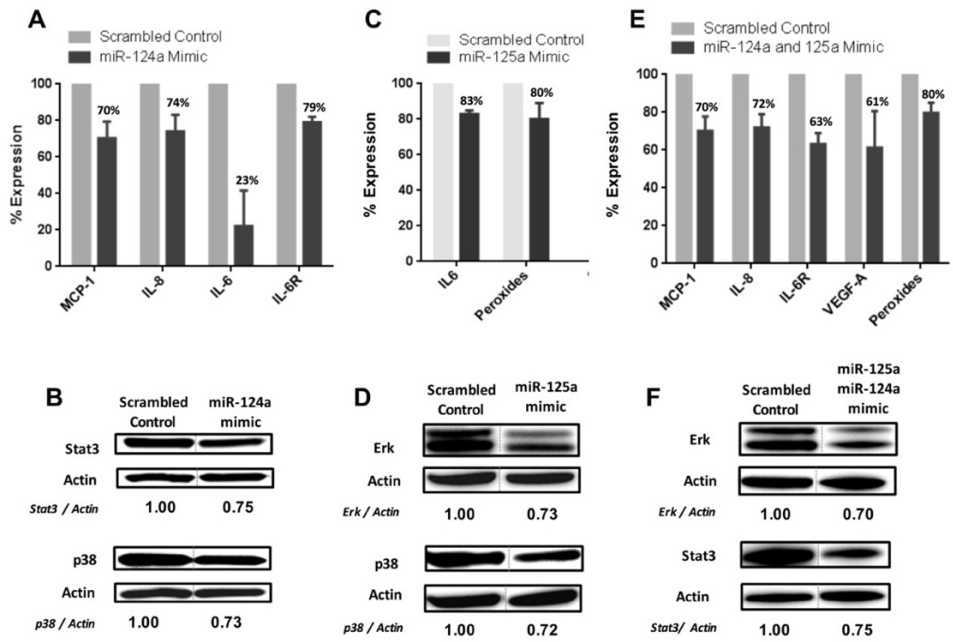

Figure 7. Transfection with miR-124a and/or -125a mimics promoted decreased expression of inflammatory markers and their intracellular pathways. Monocytes isolated from healthy donors were transfected with 100 nmol/L miR-124a, miR-125a mimics -either separately or in conjunction-, and a non-specific control (scrambled) by using siPORT^{TM} NeoFX^{TM} transfection agent following manufacturer's protocols. *Forty-two* hours after transfection cells were activated with 10 mg/mL LPS for 6h, and potential

targets were analyzed by qRT-PCR (MCP-1, IL-8, IL-6, IL-6R and VEGF), Flow cytometry (peroxides), and Western blot (STAT3, ERK, and p38MAPK). Data, obtained from 4 independent transfection experiments, were expressed as changes relative to the values of the cells transfected with scrambled control, and set as 100%. (A and B) Changes promoted by transfection of monocytes with miR-124a mimic. (C and D) Changes promoted by transfection of monocytes with miRNA mimic for miR-125a. (E and F) Changes promoted by simultaneous transfection of monocytes with miR-124a and miR-125a mimics. Western blots show representative results from 4 separated experiments with similar results. Lanes were run on the same gel under the same experimental conditions but were non-contiguous. Cropping lines are used in the figure. Full-length blots are presented in Supplementary Figure S6B for key data.

5. DISCUSSION

There is increasing evidence connecting an imbalance between various proinflammatory mediators with higher CVD risk in APS and SLE patients[31,32]. Accordingly, in previous reports, we demonstrated that prothrombotic and inflammatory molecules, oxidative stress markers, and mitochondrial dysfunction, were associated with premature atherosclerosis and/or the occurrence of thrombotic events in SLE[9,16]. Moreover, the same inflammatory/oxidative molecules seemed to orchestrate the atherothrombotic status present in APS patients[3], suggesting specificity of the CV comorbidity in these autoimmune patients. These preliminary data encouraged us to come across a new search of mechanisms orchestrating those processes, such as the study of miRNAs that specifically modulate those targets, and to analyze their expression and modulation in the setting of APS and SLE.

The present study demonstrated, for the first time, the altered expression of a number of miRNAs directly involved in atherothrombosis, and their modulation by effect of specific autoantibodies in both pathologies.

Previous studies by our group supported the occurrence of a specific gene profile in SLE patients positive for aPL in relation to those negative for aPL, which was further associated to atherothrombosis[16]. Moreover, that

study linked the presence of a high CVD risk in the formers with the overexpression of various prothrombotic and proinflammatory mediators (i.e. PAR-2, TF, MCP-1, or tPA). However, the classical inflammatory cytokines (ie, IFNγ, TNFα, IL-1β, IL-6) that orchestrate common pathophysiological processes in SLE (ie, nephritis, skin manifestations, neurological affectations, etc) were more specifically linked to aPL(-) SLE patients, suggesting molecular and cellular specificity of the CV comorbidity. The present study further confirmed this hypothesis, showing that aPL(+) SLE patients displayed an specific dysregulation of a number of miRNAs in relation to aPL-(-) SLE patients. In accordance with that results, proinflammatory profile also differed among aPL(-) and aPL(+)-SLE patients in the same way as described above, including a number of targets of the measured miRNAs. That data supported the direct linkage among the altered expression of specific microRNAs, the presence of aPL, and the increased risk of atherothrombosis in this group of SLE patients.

The analysis of the miRNA expression in SLE patients that had suffered previous thrombotic events and those without thrombosis in relation to healthy donors, demonstrated an altered expression of miRNAs independent of the thrombophilic state. In addition, those changes were directly related to the positivity for both aPL and anti-dsDNA antibodies, as indicated by association studies. Furthermore, their expression was modulated by in vitro stimulation with both autoantibodies. Thus, those miRNAs could be considered mostly markers of immune activation in lupus, which in turn might lead to a prothrombotic and proinflammatory status in SLE patients.

In line with those results, the analysis of miRNAs in patients with previous thrombotic events but without an autoimmune-related profile revealed a differential pattern of expression, with almost no changes in neutrophils, and a significant reduction in the majority of miRNAs in monocytes in relation to healthy donors. Previous studies have reported a substantial involvement of the evaluated miRNAs on CVD[33-37]. Yet, thrombotic patients displayed a specific profile of miRNA expression that would reflect a differential and specific mode of regulation and activity in relation to autoimmune patients, on which autoantibodies most probably play a key role.

Among the miRNAs selected for characterization in APS and SLE patients, miR-146a and miR-155 have been previously shown to play critical roles in lymphocyte development, differentiation, function, and also in the control of both innate and adaptive immune responses. Deregulated miR146a and miR-155 expression and/or function have been associated with various autoimmune diseases including SLE and rheumatoid arthritis (RA)[18,38]. In our hands, miR-146a and miR-155 were found downregulated in neutrophils, while their expression was found significantly increased in monocytes from both SLE and APS patients, and a trend to a reduction was observed in lymphocytes. Moreover, their overexpression correlated with levels of ACA-IgG and anti-dsDNA antibodies, as well as with increased plasma levels of pro-inflammatory molecules and oxidative stress markers. Our *in vitro* studies further confirmed the *in vivo* correlations, demonstrating that both aPL-IgG and anti-dsDNA antibodies promoted a significant reduction of these miRNAs in neutrophils and a prominent increase in monocytes.

Contrary to our results, a previous study reported that miR-146a was under expressed in PBMCs of SLE patients, and negatively correlated with type I interferon and clinical disease activity[39]. That apparently contradictory data may be related to a distinct miRNA expression in different leucocyte subsets, which might differentially contribute to the overall inflammatory status in both autoimmune diseases. Indeed, a recent study has shown that monocytes overexpressing miR-146a display a dampened inflammatory response[40] suggesting that this miRNA might play a role as a molecular brake on inflammation in the setting of APS and SLE. MiR-155 levels have been reported to be elevated in B but low in T cells from SLE patients[41] and deleting miR-155 prevents the production of harmful antibodies, alleviating lupus-like disease in mice[42]. In our study, as in the case of miR-146a, the over-expression of miR-155 found in APS and SLE monocytes might act as a protective factor against the inflammatory effect of autoantibodies, as previously reported in the setting of RA[43,44].

On the contrary, significant reduction was found in miR-125a levels in both neutrophils and monocytes from APS and SLE patients. This miRNA is involved in the inflammatory chemokine pathway of SLE, and increases the expression of inflammatory chemokine RANTES by targeting KLF13 in SLE[45]. In the present study, reduced miR-125a levels correlated with oxidative stress markers, inflammatory and prothrombotic molecules, and autoimmunity parameters, and were also related to the occurrence of thrombotic events in both APS and SLE patients. Furthermore, miR-125a overexpression in monocytes significantly reduced peroxides levels, IL-6 expression, and intracellular inflammatory signaling kinases, including ERK and p38 MAPK.

It is known that miR-124a targets monocyte chemoattractant protein 1 (MCP-1)[46], which is significantly elevated in APS, SLE, and RA patients, and involved in the CV pathogenesis of that autoimmune conditions[3,9,47]. In the present study, miR-124a was found significantly reduced in both neutrophils and monocytes from APS and SLE patients, and related to thrombotic events and a pathological CIMT. MiR-124a expression was strongly downregulated by autoantibodies from both autoimmune conditions, and its overexpression in monocytes downregulated not only MCP-1 but also IL-8, IL-6, IL-6R, STAT3 and p38 MAPK. Thus, miR-124a, together with miR-125a seems to orchestrate the inflammatory status that underlies the pathophysiology of CVD in APS and SLE. Furthermore, our overall data unveiled new roles and targets for both miRNAs in those autoimmune diseases.

Some miRNAs are emerging as being associated with or localized in mitochondria, could play a direct role in regulating mitochondrial function[48-50], and their modulation may be involved in various pathological processes, including inflammation. The present study reports an altered expression of some of these miRNAs, in APS and SLE patients, that is associated to the mitochondrial dysfunction and the pro-oxidative status present in those diseases. Thus, the expression of miR-125a, miR-155 and miR-146a correlated with the levels of peroxides as well as with the increased percentage of cells with altered $\Delta\Psi m$ and the increased activity of mitochondrial SOD. Moreover, transfection of monocytes with miR-125a mimics promoted a reduction in peroxides production. Thus, the up-regulation of those miRNAs could mediate the loss of mitochondrial integrity and function, thus contributing to the pro-oxidative status of APS and SLE.

In general, APS and SLE patients are treated with anticoagulants/anti-agregants, antimalarial agents, corticosteroids, and immunosuppressive medications. All of them have been shown to influence miRNA expression, an epigenetic mechanism that might help to delineate the mechanisms underlying their effects. Thus, studies have demonstrated that HCQ altered the expression of miR-21 and miR-let-7a in PBMCs from NZB/W lupus mice, partially explaining its anti-inflammatory effects[51]. Similarly, in NZB/W mice treated with prednisone, expression levels of miR-lt-7a, miR 21, miR 146a and miR155 all changed[48]. Also, immunosuppressants such as methotrexate have been confirmed to alter the epigenetic status in other diseases, such as cancer[52]. With that premises, in our cohort of patients, the putative effects of the administered drugs on the expression of the miRNAs evaluated in APS and SLE patients were assessed. As a general feature, we found a high homogeneity in the treatments administered, so that most APS patients were treated with anti-agregants/anticoagulants and antimalarials, while almost all the SLE patients received A/A, HCQ and prednisone. We could also identify a number of SLE patients further treated with immunosuppressive drugs. Comparative analyses demonstrated a significant increase in the expression of miR146 and miR155 in monocytes of SLE patients receiving immunosuppressive therapy in relation to the ones non-treated with those drugs. As stated above, monocytes in our cohort of SLE patients showed a significant overexpression of both miR146 and miR155. That overexpression was hypothesized to act as a protective factor against the inflammatory effects of autoantibodies. Due to the anti-inflammatory nature of immunosuppressive drugs, it might be speculated that the even

increased expression of both miRNAs in immunosuppressant-treated patients could further contribute to repress inflammation.

A remarkable heterogeneity in autoantibody profile and clinical presentation is well known in SLE patients. The two prominent classes of autoantibody populations in SLE are targeted against either dsDNA or RNA-associated proteins. However, the basis for this distinctive autoantibody profile and its regulation in SLE patients is poorly understood. In a previous study, we demonstrated that the occurrence of thrombotic events in SLE was associated with factors related to autoimmunity, including titers of aCL-IgG and anti-dsDNA[9]. By using an *in vitro* approach, in the present study we have demonstrated that anti-dsDNA antibodies not only paralleled the altered appearance of some proinflammatory mediators in SLE, but also acted as direct modulators of the expression of a number of miRNAs that, in turn, orchestrated their expression. Our results were further supported by a recent study that reported a differential and varying miRNA expression profile in subsets of SLE patients characterized on the basis of distinct autoantibodies repertoires. Using the QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity), they identified cell cycle- and cytoskeleton remodeling-related events as the main targets of miRNAs dysregulated in anti-ENA+ patients, whereas miRNAs dysregulated in anti-dsDNA+ patients were found to be involved in multiple cytokine signaling pathways. Our study further delineated that association[53].

miRNAs expressed in the vasculature play important roles in CVD[54]. Vascular inflammation is an early step in atherogenesis, and many miRNAs

are induced in inflamed ECs. Thus, previous studies reported that TNFα treatment decreased miR181b expression (promoting induction of adhesion molecules such as VCAM-1)[55] and induce miR-17, miR31, miR155, miR-221 and miR222 expression (regulating the adhesion of neutrophils and T cells to activated ECs, as well as the proliferation and migration of ECs)[56,57]. The present study further showed that aPL-IgG and anti-ds-DNA-IgG autoantibodies downregulated in ECs all the miRNAs evaluated, also related to vascular dysfunction in the setting of APS and SLE, as demonstrated by the induced upregulation of MCP-1, TF, and VCAM-1, and the downregulation of eNOS in ECs. Thus, analyzed miRNAs might also play a pivotal role on endothelial function in both autoimmune conditions.

The generation of miRNAs is mainly dependent on the RNAse III enzyme Dicer, the levels of which vary in different normal cells and in disease states[58]. It has been shown that interruption of miRNA biogenesis machinery (through spontaneous or induced Dicer-deficiency) contributes to the abnormal T and B cell development, as well as to altered endothelial function, leading to vascular dysfunction and systemic autoimmune diseases[59]. In this study, we further demonstrated a reduced expression of Dicer, along with other proteins related to miRNA biogenesis, in neutrophils from APS and SLE patients. That reduced expression was associated with clinical aspects of these diseases, including the occurrence of thrombotic events and the presence of a pathologic CIMT. Moreover, the analysis of a wide set of miRNAs in neutrophils from SLE patients demonstrated the underexpression of approximately an 80% of them when compared with neutrophils from healthy donors, thus supporting

the hypothesis that interrupted miRNA biogenesis plays a key role in SLE development and progression.

Previous studies demonstrated that Dicer protein expression can be inhibited by multiple types of stress, including reactive oxygen species, phorbol esters and the Ras oncogenes, as well as IFN type I[60]. Accordingly, the presence of a chronic inflammatory and oxidative status in APS and SLE patients might contribute to the reduced levels of Dicer found in neutrophils. Our *in vitro* studies further demonstrated that Dicer mRNA and protein expression levels, in both neutrophils and endothelial cells, were downregulated by effect of specific autoantibodies of APS and SLE, which are as well responsible for the altered expression of proinflammatory and oxidative stress markers. Those molecules in conjunction might thus influence Dicer inhibition.

Transfection studies in monocytes with miR-124a and/or miR-125a showed a downregulation of a number of inflammatory markers associated with the pathophysiology of CVD in APS and SLE. Moreover, we noticed that the simultaneous transfection with both miRNAs did not potentiate the effect caused by each miRNA administered separately, but increased the number of molecules targeted. Of note, the inhibitory effect was moderate. It has been widely demonstrated that while a single miRNA may target hundreds of genes, the effect of miRNAs on individual target protein synthesis is mild and moderate[56]. However, given that a specific inflammatory-related-gene may contain targeting sites for different miRNAs, it is plausible that multiple SLE/APS-associated miRNAs, rather than a single miRNA, synergistically act together to alter the overall inflammatory status in these patients.

Taken together, the current study has revealed that: 1. Specific miRNAs might act as potential biomarkers of immune activation and atherothrombosis in APS and SLE patients. 2. miRNA biogenesis is significantly altered in neutrophils of APS and SLE patients and associated to both, the presence of a pathologic CIMT, and the occurrence of thrombotic events. 3. Anti-dsDNA and aPL antibodies regulate CVD in APS and SLE, at least partially, by modulating the biogenesis and the expression of miRNAs.

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Contributions

C.P.-S., P.R.-L., R.T., and I.A-R., developed the *in vivo* assays, performed the experiments, solved technical problems and drafted the manuscript; M.A.A., F.V., and P.S., followed up with patients, revised the manuscript, and contributed useful discussion; A.R.A., C.M., R.G.-C., N.B., and Ch.L-P. formed the hypothesis, directed and coordinated the project, designed the experiments, analyzed the data, and wrote the manuscript; E.C.-E. and M.J.C performed clinical analysis, revised the manuscript, and contributed useful suggestions; Y.J.G. performed statistical analysis, helped to draft the manuscript, and discussed results. All authors read and approved the manuscript.

Competing interests

The authors declare no competing financial interests.

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7. SUPPLEMENTARY INFORMATION

Flow cytometry analyses, and analysis of oxidative stress biomarkers in purified leukocytes and plasma, and western blotting.

Flow cytometry analysis

Flow cytometric analysis was performed in monocytes as previously described [6], using a FACScan (BD Biosciences, San Jose, CA,) and monoclonal anti-human FITC-conjugated TF antibodies (clone TF9-6B4, American Diagnostica, Greenwich, CT,), and anti-human FITC-conjugated PAR2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The BD Cytofix/Cytoperm fixation/permeabilization kit was used to analyse the intracellular expression of some cytokines (i.e. IL-6, IL-8, MCP-1), according to manufacturer's instructions.

FlowCytomix

CD40L, IFNα, IFNγ, IL-1, IL-2, IL-6, IL-8, IL-10, IL-17, IL-23, MCP-1, MIP-1α, TNFα, tPA, VEGF-A, and sP-selectin levels were quantified in sera using a cytofluorimetry-based ELISA system (Flowcytomix, Bender Medsystem GmbH, Austria). Two-colour cytometric analysis was performed using FACScalibur cytometer (Becton Dickinson Immunocytometry Systems (BDIS); San José, CA). Data were obtained and analysed using the FlowCytomix Pro software.

Determination of oxidative stress biomarkers in purified leukocyte subsets

Oxidative stress biomarkers were analysed in purified leukocytes using a dual-laser FACSCalibur (Becton Dickinson, Mountain View, CA). Test standardization and data acquisition analysis were performed using the

CELL Quest software (Becton Dickinson, Mountain View, CA). A forward and side scatter gate was used for the selection and analysis of the different cell subpopulations.

For the assessment of ROS generation, including the joint detection of peroxides and peroxynitrites, cells were incubated with 5 μM DHR-123 at 37°C for 30 min in the dark. The JC-1 Mitoscreen assay (Becton Dickinson, Mountain View, CA) was used (final concentration 2 μM) to assess the mitochondrial membrane potential ($\Delta\psi_m$) according to manufacturer's instructions.

Determination of cell oxidative stress biomarkers

Mitochondrial SOD activity (Mn-SOD), Catalase (CAT) activity and Glutathione peroxidase (GPx) activity were assayed in cell lysates using specific kits (Cayman Chemical Company, ciudad, MI) according to manufacturer's instructions.

Western blotting

Dicer, p38 MAPK, Erk, STAT-3, GAPDH and actin protein levels were determined by Western blotting [6], using specific antibodies (Abcam and Santa Cruz Biotechnology).

RNA isolation and qRT-PCR for mRNA and microRNA expression

Total RNA from lymphocytes, monocytes, and neutrophils was extracted using TRI Reagent (Sigma, St Louis, MO) following the manufacturer's recommendations. The integrity of RNA was verified by optical density (OD) absorption ratio OD260/OD280 between 1.7 and 1.8.

For first strand cDNA synthesis, 1μg of total RNA was reverse transcribed using random hexamers (Roche Diagnostic, Indianapolis, IN) as primers

and Transcriptor Reverse Transcriptase (Roche Diagnostic, Indianapolis, IN). Gene expression was assessed by real time PCR using a LightCycler Thermal Cycler System (Roche Diagnostics, Indianapolis, IN,). The reaction was performed, following the manufacturers protocol, in a final volume of 25µl.

The reactions consisted of an initial denaturing of 10 min at 95 ºC, then 40 cycles of 15 seconds denaturing phase at 95ºC, and 1 minute annealing and extension phase at 60ºC. A threshold cycle (Ct value) was obtained for each amplification curve and a ΔCt value was first calculated by subtracting the Ct value for human glyceraldehyde-3-phosphate dehydrogenase cDNA from the Ct value for each sample and transcript. Fold changes compared with the endogenous control were then determined by calculating 2-ΔCt. Every sample was performed in triplicate and negative controls were included in all the reactions. Test reproducibility for all investigated transcripts was less than 0.5% in inter-test experiments and even lower in intra-test experiments. MiRNA biogenesis modulators Dicer, Drosha, Argonaute-1, Argonaute-2, Exportin-5, as well as a number of inflammatory molecules were quantified.

For quantification of mature miRNA levels in lymphocytes, neutrophils and monocytes trizol purified RNA was used. cDNA was synthesized from 200 ng RNA using individual miRNA-specific RT primers contained in the TaqMan® MicroRNA Reverse Transcription Kit. Each cDNA was amplified using the TaqMan® MicroRNA assays together with TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Life Technologies, Madrid, Spain).

The $2^{-\Delta Ct}$ method was used to calculate the relative abundance of miRNAs compared with U6 snRNA expression and set as 100%.

NanoString sample preparation and data analysis.

A pool with RNA purified of neutrophils from 5 SLE patients and other pool with RNA purified of neutrophils from 5 healthy donors was performed. A total of 100 ng RNA input was used per sample and conditions were set according to the manufacturer's recommended protocol (NanoString Technologies; Seattle, WA). Briefly, mature miRNAs were ligated to a species-specific tag sequence (miRtag) via a thermally controlled splinted ligation. The unligated miRtags were removed with enzymatic purification, and miRtagged mature miRNAs were then hybridized with an nCounter Human (V2) miRNA Expression Assay CodeSet overnight at 65°C. The unhybridized CodeSet was removed with automated purification performed on an nCounter Prep Station, and the remaining target:probe complexes were transferred and bound to an imaging surface. Counts of the reporter probes were tabulated for each sample by the nCounter Digital Analyzer, and raw data output was imported into nSolver (http:// www.nanostring.com/products/nSolver). The nCounter® program uses a normalization factor to account for technical noise (such as variations in hybridization, purification, binding efficiency). Data were normalized by the genometric mean of all targets using the nSolver software. In addition, six internal negative controls and six positive controls were included in the nCounter miRNA Expression assays, to correct for background noise. The mean of the negative controls served as the medium stringency threshold for miRNA detection and high stringency was calculated by adding 2SD to the mean of the negative controls (threshold= mean+ 2SD).

Purification of anti-dsDNA IgG from SLE patients

Anti-dsDNA IgG antibodies from the pooled sera of 7 SLE patients (characterised by high titres of anti-dsDNA) were purified using a commercial kit (Quanta Lite-INOVA Diagnostics, San Diego, CA). Briefly, 100 μl of pooled sera were added to the microtitres plates coated with highly purified calf thymus ds DNA which allowed the binding of the antibodies (anti-ds-DNA). Next, anti-ds-DNA were eluted with 100 μl/well 0.1 M glycine/0.3 M NaCl pH 2.8 for 20 minutes at room temperature, then neutralised with 2M Tris-HCl pH 7.6 solution. Thereafter, the eluates were concentrated using Amicon Ultra-0.5 centrifugal filter devices from Merck Millipore (Darmstadt, Germany) following the manufacturer's recommendations. To assess the anti-dsDNA activity of these samples, 1:100 diluted samples were tested using the same commercial kit utilized for anti-dsDNA isolation (INOVA Diagnostics) according to the manufacturer's instructions.

Cell transfection

Monocytes purified from healthy donors were plated 24 hours before transfection in 6-well plates with complete medium without antibiotics (Opti-MEM, Life Technologies, Madrid, Spain). Cells were transfected with 100 nmol/L miRNA mimic (Life Technologies, Madrid, Spain) for miR-124a, miR-125a -either separately or in conjunction-, and a non-specific control (scrambled) by using siPORT™ NeoFX™ transfection agent (Life Technologies, Madrid, Spain) following manufacturer's protocols. *Forty-two* hours after transfection cells were activated with 10 mg/mL LPS (Sigma-Aldrich, Madrid, Spain) for 6h, and potential targets were analyzed by qRT-PCR, Flow cytometry, Flowcytomix, and Western blot. Data were

expressed as changes relative to the values of the cells transfected with scrambled control, and set as 100%.

Supplementary Tables

Supplementary Table SI. Parameters related to inflammation and oxidative stress in APS and SLE patients.

	Healthy donors (N=56)	APS patients (N=23)	P#	SLE patients (N=65)	P#
INFLAMMATORY PARAMETERS*					
Tissue Factor (%)	15.54±11.67	33.51±30.51	0.030	24.87±20.66	0.011
PAR2 (%)	5.34±6.24	15.31±21.39	0.007	13.31±20.02	0.020
sCD40L(ng/mL)	16.06±15.57	18.10±15.64	n.s.	11.63±11.37	n.s.
IFN-α (pg/mL)	119.44±245.55	53.19±150.44	n.s	109.47±171.62	n.s.
IFN-γ (pg/mL)	4.72±11.42	0	n.s.	0.39±1.09	n.s.
IL-1ß (pg/mL)	12.98±21.26	9.38±16.25	n.s	18.05±34.05	n.s.
IL-2 (pg/mL)	26.97±38.44	0	n.s.	184.80±290.46	n.s.
IL-6 (pg/mL)	0.81±1.39	1.87±2.56	n.s.	0.48±0.91	0.001
IL-8 (pg/mL)	4.06±1.80	6.48±2.80	0.024	27.38±45.12	0.001
IL-10 (pg/mL)	23.36±65.16	29.31±77.54	n.s.	0.93±1.43	n.s.
IL-17 (pg/mL)	4.78±15.84	--	--	53.51±91.89	0.024
IL-23 (pg/mL)	0	--	--	44.48±83.63	0.021
MCP-1 (pg/mL)	59.81±18.08	96.76±72.44	0.040	192.28±195.99	0.001
MIP1-α (pg/mL)	249.93±343.70	463.19±437.91	n.s.	353.01±503.34	n.s.
sP-Sel (pg/mL)	196.15±75.32	188.99±67.06	n.s.	158.68±50.21	n.s.
TNF-α (pg/mL)	0	0	--	0.30±0.68	n.s.
t-PA (ng/mL)	1,79±0.63	2,60±1,49	0.023	3,83±2,36	0.001
VEGF-A (pg/mL)	95.18±55.16	101.66±107.19	n.s.	100.38±78.28	n.s.
OXIDATIVE STATUS*					
Peroxides/Peroxinytrites (MIF)$					
Monocytes	36.62±12.39	61.17±32.17	0.031	48.21±27.55	0.025
Neutrophils	10.62±5.92	13.69±7.81	0.048	13.70±6.53	0.049
Mitochondrial membrane potential (% positive cells)					
Monocytes	4.62±6.10	20.61±21.74	0.022	12.35±13.05	0.001
Neutrophils	3.18±7.13	11.22±17.69	0.019	14.07±21.15	0.002
Manganese-SOD (U/min/mL/µg prot)					
Monocytes	0.08±0.05	0.30±0.20	0.001	0.25±0.20	0.042
Neutrophils	2.41±1.13	2.10±0.62	n.s.	2.03±1.13	n.s.
Catalase (nmol/min/mL/µg protein)					
Monocytes	217.49±103.27	98.68±42.27	0.019	217.62±146.08	n.s.
Neutrophils	571.40±404.42	297.18±101.11	0.001	386.28±188.62	0.033
GPx (nmol/min/mL/µg protein)					
Monocytes	2.10±0.77	1.13±0.87	0.049	1.89±0.34	n.s.
Neutrophils	0.50±0.19	0.26±0.15	0.001	0.33±0.19	0.004

* Values are mean ± SD.

#P<0.05 vs healthy donors.

$Unless specified, data are referred to monocytes.

Supplementary Table SII. Correlations/Associations among microRNAs, oxidative stress, inflammation and autoimmunity parameters in APS patients.

	APS PATIENTS			
Neutrophils	**OXIDATIVE STRESS***	**INFLAMMATION***	**AUTOIMMUNITY#**	
Hsa-miR-124a	**Catalase** 0.460/0.009			**ACA-IgG positivity** *(P=0.015)* Neg. 87.85 ± 182.08 Pos. 6.58 ± 10.73
Hsa-miR-125a	**Catalase** 0.494/0.004		**LA positivity** *(P=0.004)* Neg. 95.03 ± 138.70 Pos. 16.55 ± 22.42	
Hsa-miR-125b	**Catalase** 0.544/0.001	**CRP** -0.315/0.024	**LA positivity** *(P=0.002)* Neg. 95.68 ± 122.49 Pos. 19.93 ± 23.46	
Hsa-miR-146a	**Catalase** 0.384/0.030	**CRP** -0.323/0.021	**LA positivity** *(P=0.004)* Neg. 95.65 ± 81.74 Pos. 24.59 ± 33.14	
Hsa-miR-155	**Catalase** 0.525/0.002	**CRP** -0.460/0.001	**LA positivity** *(P=0.001)* Neg. 96.08 ± 68.60 Pos. 23.55 ± 23.18	
Hsa-miR-222	**Catalase** 0.464/0.007	**IL-8** -0.480/0.020		**ACA-IgG positivity** *(P=0.006)* Neg. 85.99 ± 101.17 Pos. 27.61 ± 18.45
Monocytes	**OXIDATIVE STRESS***	**INFLAMMATION***	**AUTOIMMUNITY#**	
Hsa-miR-124a			**LA positivity (P=0.004)** Neg. 95.03 ± 138.70 Pos. 16.55 ± 22.42	
Hsa-miR-125a	**ΔΨm** -0.466/0.014	**CRP** -0.287/0.045 **TF** -0.449/0.008 **PAR-2** -0.507/0.002		**ACA-IgG positivity (P=0.001)** Neg. 84.30 ± 109.08 Pos. 5.06 ± 3.53
Hsa-miR-146a	**SODmit** 0.733/0.016	**IL-8** 0.423/0.031		
Hsa-miR-155	**ΔΨm** 0.459/0.014 **GPx** -0.580/0.048	**CRP** 0.432/0.002 **IL-8** 0.468/0.021		

CRP: C-reactive protein; TF: tissue factor expression; PAR2: Protease activated receptor type 2; SODmit: superoxide dismutase mitochondrial; ΔΨm: mitochondrial membrane potential; GPx: glutathione peroxidase.

*Correlation studies: Data are expressed as Spearman Rho coeficient / P value.

#Association studies: Data are expressed as the mean ± SD. *P* value in brackets.

Supplementary Table SIII. Correlations/Associations among microRNAs, oxidative stress, inflammation and autoimmunity parameters in SLE patients.

	SLE PATIENTS			
Neutrophils	**OXIDATIVE STRESS***	**INFLAMMATION***	**AUTOIMMUNITY#**	
Hsa-miR-124a	**Catalase** (0.340/0.046)	**IL-23** (-0.435/0.049)	**Anti-dsDNA positivity** *(P= 0.001)* Neg. 69.84±155.66 Pos. 2.41±2.41	**ACA-IgG positivity** *(P=0.007)* Neg. 75.83±161.47 Pos. 7.06±11.17
Hsa-miR-125a	**Peroxides** (-0.313/0.025) **ΔΨm** (-0.292/0.049)	**IL-6** (-0.519/0.001) **IL-8** (-0.369/0.029)		**ACA-IgG positivity** *(P=0.001)* Neg. 81.41±77.08 Pos. 23.16±25.39
Hsa-miR-125b	**Catalase** (0.420/0.011)	**IL-23** (-0.441/0.040)		
Hsa-miR-146a		**t-PA** (-0.373/0.027)	**Anti-dsDNA positivity** *(P= 0.002)* Neg. 85.15±82.83 Pos. 24.77±21.07	
Hsa-miR-155	**Catalase** (0.403/0.015) **Peroxides** (-0.280/0.046)	**CRP** (-0.341/0.005) **IL-6** (-0.453/0.005) **IL-8** (-0.371/0.026) **t-PA** (-0.322/0.049)		**ACA-IgG positivity** *(P=0.043)* Neg. 93.62±80.48 Pos. 42.96±45.63
Hsa-miR-222	**Catalase** (0.356/0.033)	**IL-6** (-0.388/0.019) **IL-17** (-0.375/0.048) **IL-23** (-0.423/0.044)	**Anti-dsDNA positivity** *(P= 0.003)* Neg. 77.03±92.55 Pos. 27.61±19.81	**ACA-IgG positivity** *(P=0.003)* Neg. 78.74±93.50 Pos. 26.56±24.51
Monocytes	**OXIDATIVE STRESS***	**INFLAMMATION***	**AUTOIMMUNITY#**	
Hsa-miR-125a		**CRP** (-0.281/0.027) **TF** (-0.540/0.001) **PAR-2** (-0.493/0.001)		
Hsa-miR-146a		**IL-17** (0.644/0.049)	**Anti-dsDNA positivity** Neg. 123.20±129.03 Pos. 259.37±212.18 *(P= 0.039)*	**ACA-IgG positivity** Neg. 128.86±131.81 Pos. 300.77±200.45 *(P=0.009)*
Hsa-miR-155	**ΔΨm** (0.342/0.020)	**CRP** (0.315/0.013) **IL-23** (0.649/0.042)	**Anti-dsDNA positivity** Neg. 133.02±126.44 Pos. 263.07±216.88 *(P= 0.046)*	**ACA-IgG positivity** Neg. 146.13±144.42 Pos. 299.24±227.89 *(P=0.034)*

CRP: C-reactive protein; TF: tissue factor expression; PAR2: Protease activated receptor type 2; SODmit: superoxide dismutase mitochondrial; ΔΨm: mitochondrial membrane potential; GPx: glutathione peroxidase.

*Correlation studies: Data are expressed as Spearman Rho coeficient / P value.

#Association studies: Data are expressed as the mean ± SD. *P* value in brackets.

Supplementary Table IV

Individual treatments in primary APS patients

ASA: Aspirin; HCQ: hydroxychloroquine; the hyphens denote not available information; NA: not applicable.

Patient	Age (years)	Sex	Arterial Thrombosis	Venous Thrombosis	Pregnancy morbidity	Carotid Intimate Media thickness	Anticoagulants/ Antiplatelets	Antimalarials (HCQ)	Corticosteroids (Prednisone)
1	46	M	YES	NO	-	Pathological	ASA	YES	NO
2	35	F	NO	YES	YES	Normal	ASA	NO	NO
3	57	F	YES	NO	NO	Normal	Acenocoumarol	NO	NO
4	36	F	NO	NO	YES	Normal	NO	NO	NO
5	61	M	YES	NO	NA	Pathological	Acenocoumarol	NO	NO
6	34	F	YES	NO	NO	Normal	Acenocoumarol	YES	NO
7	37	F	NO	NO	YES	Normal	ASA	NO	NO
8	38	F	NO	NO	YES	Normal	ASA	NO	NO
9	71	M	YES	YES	NA	Pathological	ASA	NO	NO
10	49	F	YES	NO	NO	Pathological	ASA	NO	NO
11	57	F	YES	YES	YES	Normal	Acenocoumarol	YES	YES
12	70	F	YES	NO	NO	Pathological	Acenocoumarol	NO	NO
13	36	F	YES	NO	YES	-	Acenocoumarol	NO	NO
14	35	F	YES	NO	NO	Normal	ASA	NO	NO
15	32	M	YES	NO	NA	Normal	ASA	NO	NO
16	36	M	YES	NO	NA	Normal	ASA	NO	NO
17	40	F	NO	YES	YES	Normal	Acenocoumarol	YES	YES
18	50	M	YES	NO	NA	Pathological	Acenocoumarol	NO	NO
19	56	F	YES	NO	NO	Pathological	Acenocoumarol	NO	NO
20	41	F	NO	NO	YES	Normal	ASA	NO	NO
21	44	F	YES	NO	YES	Normal	Acenocoumarol	NO	NO
22	73	F	NO	YES	NO	Pathological	ASA	YES	NO
23	48	F	YES	NO	NO	Pathological	Acenocoumarol	NO	NO

Supplementary Table V

Individual treatments in SLE patients positive for aPL

ASA: Aspirin; HCQ: hydroxychloroquine; the hyphens denote not available information; NA: not applicable.

Patient	Age (years)	Sex	Arterial Thrombosis	Venous Thrombosis	Pregnancy morbidity	Carotid Intimate media thickness	Anticoagulants/ Antiplatelets	Antimalarials (HCQ)	Immunosuppresant drugs	Corticosteroids (Prednisone)
1	39	M	NO	YES	NA	Normal	Acenocoumarol	YES	NO	NO
2	53	F	NO	YES	YES	Pathological	ASA	YES	NO	YES
3	45	F	YES	NO	NO	Normal	Acenocoumarol	NO	NO	YES
4	31	F	YES	NO	YES	Normal	ASA	YES	NO	YES
5	32	F	NO	YES	NO	Normal	ASA	YES	NO	YES
6	54	F	YES	YES	YES	Normal	Acenocoumarol	NO	NO	YES
7	47	F	YES	NO	NO	Pathological	Acenocoumarol	YES	YES	YES
8	71	F	YES	YES	NO	Pathological	Acenocoumarol	YES	NO	YES
9	48	M	YES	NO	NA	Pathological	ASA	YES	YES	YES
10	35	F	YES	NO	YES	-	Acenocoumarol	YES	NO	YES
11	41	F	YES	YES	NO	Normal	ASA	YES	NO	YES
12	57	F	YES	NO	NO	Pathological	Acenocoumarol	NO	NO	YES
13	41	F	NO	NO	NO	Normal	ASA	YES	YES	YES
14	59	F	YES	YES	YES	Pathological	Acenocoumarol	NO	NO	YES
15	48	F	YES	NO	NO	Pathological	Acenocoumarol	NO	NO	NO
16	36	F	YES	NO	NO	Normal	ASA	YES	NO	YES
17	52	F	NO	YES	NO	Pathological	Acenocoumarol	YES	NO	YES
18	43	M	NO	YES	NA	Normal	Acenocoumarol	YES	NO	YES
19	49	F	YES	NO	YES	Pathological	ASA	YES	NO	YES
20	53	F	YES	NO	NO	-	Acenocoumarol	NO	NO	NO
21	54	F	YES	NO	YES	Normal	ASA	YES	NO	YES
22	40	F	YES	NO	NO	Normal	Acenocoumarol	YES	NO	YES
23	51	F	YES	NO	NO	Pathological	Acenocoumarol	NO	NO	YES

Supplementary Table VI

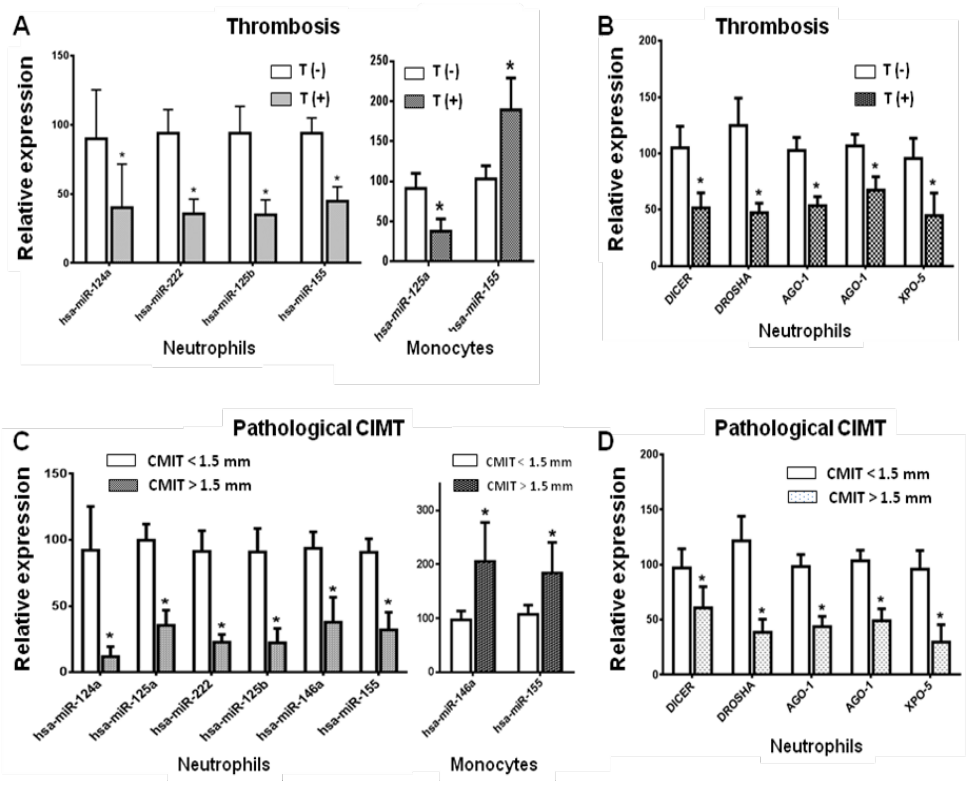
Individual treatments in SLE patients negative for aPL

ASA: Aspirin; HCQ: hydroxychloroquine; the hyphens denote not available information; NA: not applicable.

Patient	Age (years)	Sex	Arterial Thrombosis	Venous Thrombosis	Pregnancy morbidity	Carotid Intimate Media thickness	Anticoagulants/ Antiplatelets	Antimalarials (HCQ)	Corticosteroids (Prednisone)	Immunosuppressant drugs
1	27	F	YES	NO	NO	Normal	Acenocoumarol	YES	YES	YES
2	43	F	NO	NO	NO	Normal	ASA	YES	YES	NO
3	32	F	NO	NO	NO	Normal	ASA	YES	YES	NO
4	51	F	YES	NO	NO	Normal	ASA	YES	YES	NO
5	53	F	YES	NO	NO	Normal	ASA	YES	YES	NO
6	73	F	NO	NO	NO	Pathological	Acenocoumarol	YES	YES	NO
7	29	F	NO	NO	NO	Normal	ASA	YES	YES	NO
8	30	M	NO	NO	NA	Normal	NO	NO	YES	SI
9	45	F	NO	NO	NO	Normal	ASA	YES	YES	NO
10	45	F	NO	NO	NO	Normal	ASA	YES	YES	NO
11	32	F	NO	NO	NO	Normal	ASA	YES	YES	NO
12	40	M	NO	NO	NA	Normal	NO	YES	YES	NO
13	42	F	NO	NO	NO	Pathological	ASA	YES	YES	NO
14	31	F	NO	NO	NO	Normal	NO	NO	NO	NO
15	45	F	NO	NO	NO	Pathological	NO	YES	NO	NO
16	41	F	NO	NO	NO	-	ASA	YES	YES	YES
17	20	F	NO	NO	NO	Normal	ASA	YES	YES	YES
18	50	F	NO	NO	NO	Normal	ASA	YES	YES	NO
19	36	F	NO	NO	NO	-	NO	YES	YES	NO
20	45	F	NO	NO	NO	Normal	NO	YES	NO	NO
21	29	F	NO	NO	NO	Normal	ASA	YES	YES	NO
22	28	F	NO	NO	NO	-	ASA	YES	YES	NO
23	25	F	NO	NO	NO	Normal	ASA	YES	NO	NO
24	60	F	NO	NO	NO	Pathological	ASA	YES	YES	NO
25	35	F	NO	NO	NO	Normal	NO	YES	YES	YES
26	29	F	NO	NO	NO	Normal	ASA	YES	YES	YES
27	57	M	YES	NO	NA	Pathological	ASA	YES	NO	NO
28	29	M	NO	NO	NA	Normal	NO	YES	YES	YES
29	46	F	NO	NO	NO	Normal	NO	YES	YES	YES
30	37	F	NO	NO	NO	Normal	ASA	YES	YES	YES
31	27	F	NO	NO	NO	-	ASA	YES	YES	YES
32	41	F	NO	NO	NO	Normal	ASA	YES	YES	NO
33	26	F	NO	NO	NO	Normal	NO	YES	YES	NO
34	53	F	NO	NO	NO	Normal	ASA	YES	YES	YES
35	64	F	NO	NO	NO	Pathological	ASA	YES	YES	YES
36	35	F	NO	NO	NO	Normal	ASA	YES	YES	NO
37	18	M	NO	NO	NA	Normal	NO	NO	NO	NO
38	24	F	NO	NO	NO	Normal	NO	YES	YES	NO
39	28	F	NO	NO	NO	Normal	ASA	YES	YES	NO
40	48	F	NO	NO	NO	Pathological	ASA	YES	YES	YES
41	35	F	NO	NO	NO	Normal	ASA	YES	YES	NO

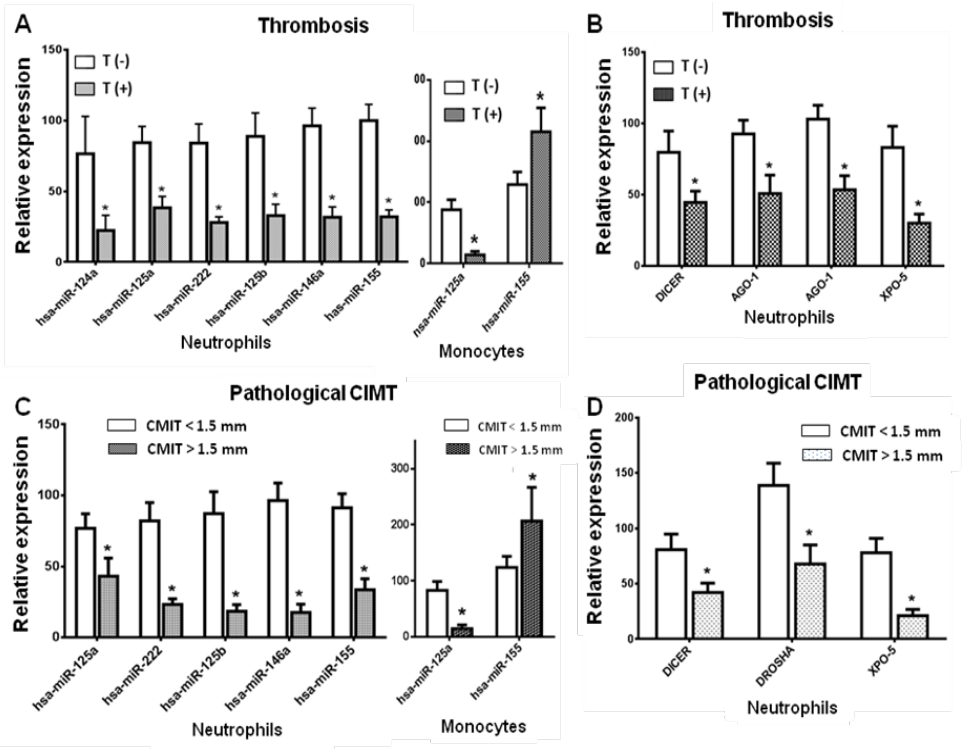
Supplementary Figures

Supplementary Figure S1



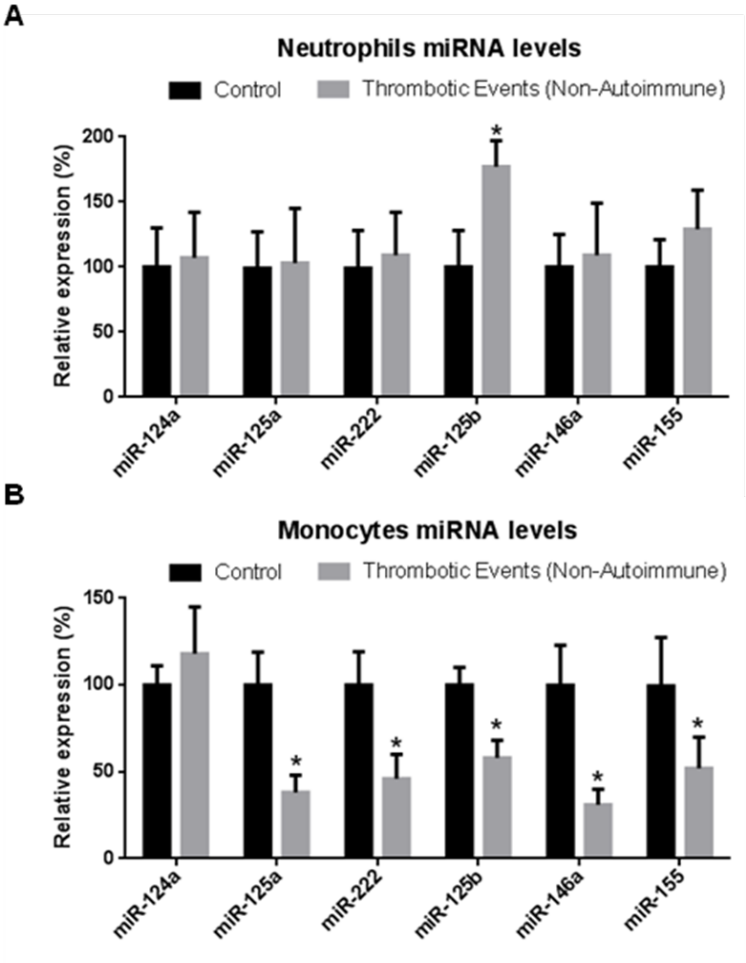
Supplementary Figure S1. Association studies among miRNAs or biomarkers of miRNA biogenesis expression levels and increased carotid intima-media thickness (CIMT) or occurrence of thrombotic events (T) in SLE patients. (A) Relationship between the occurrence of thrombotic events and the expression of the selected miRNAs in neutrophils and monocytes. (B) Relationship between the occurrence of thrombotic events and the expression of various biomarkers of miRNA biogenesis in neutrophils. (C) Relationship between the presence of increased CIMT and the expression of the selected miRNAs in neutrophils and monocytes. (D) Relationship between the presence of increased CIMT and the expression of various biomarkers of miRNA biogenesis in neutrophils. Significant differences versus patients without thrombosis or versus patients without increased CIMT (*P<0.05).

Supplementary Figure S2



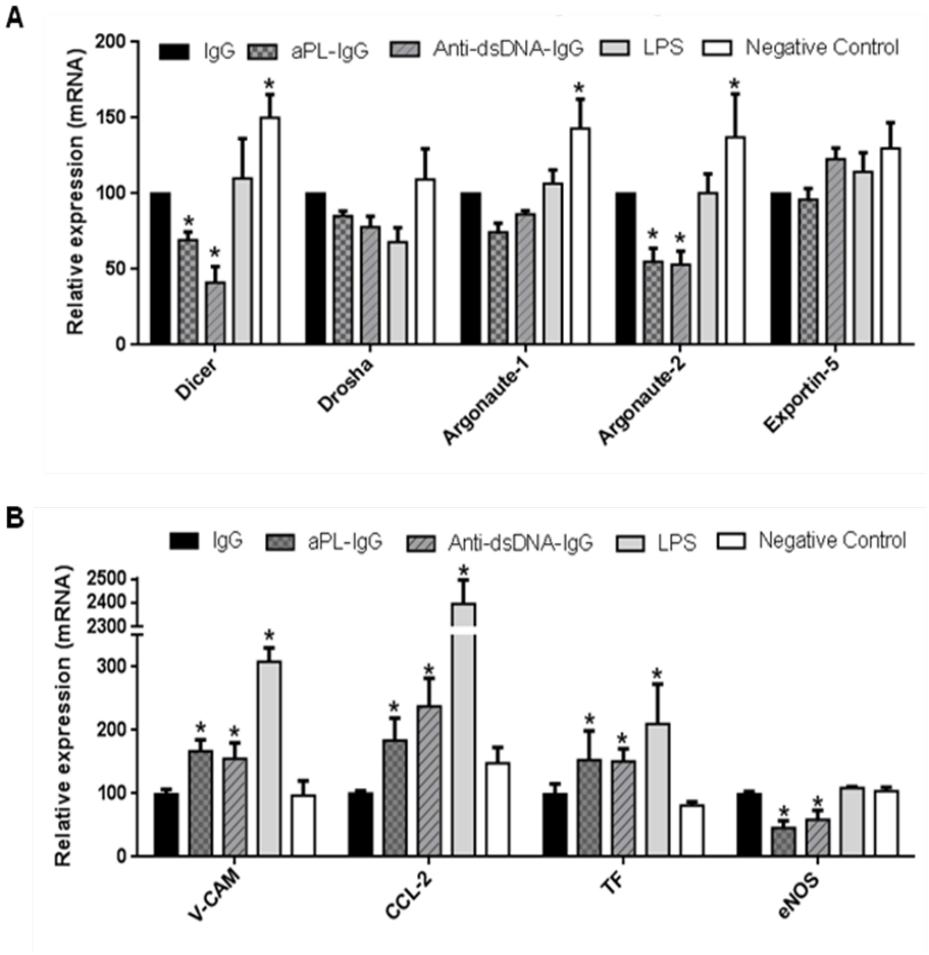
Supplementary Figure S2. Association studies among miRNAs or biomarkers of miRNA biogenesis expression levels and increased carotid intima-media thickness (CIMT) or occurrence of thrombotic events (T) in primary APS patients. (A) Relationship between the occurrence of thrombotic events and the expression of the selected miRNAs in neutrophils and monocytes. (B) Relationship between the occurrence of thrombotic events and the expression of various biomarkers of miRNA biogenesis in neutrophils. (C) Relationship between the presence of increased CIMT and the expression of the selected miRNAs in neutrophils and monocytes. (D) Relationship between the presence of increased CIMT and the expression of various biomarkers of miRNA biogenesis in neutrophils. Significant differences versus patients without thrombosis or versus patients without increased CIMT (*P<0.05).

Supplementary Figure S3



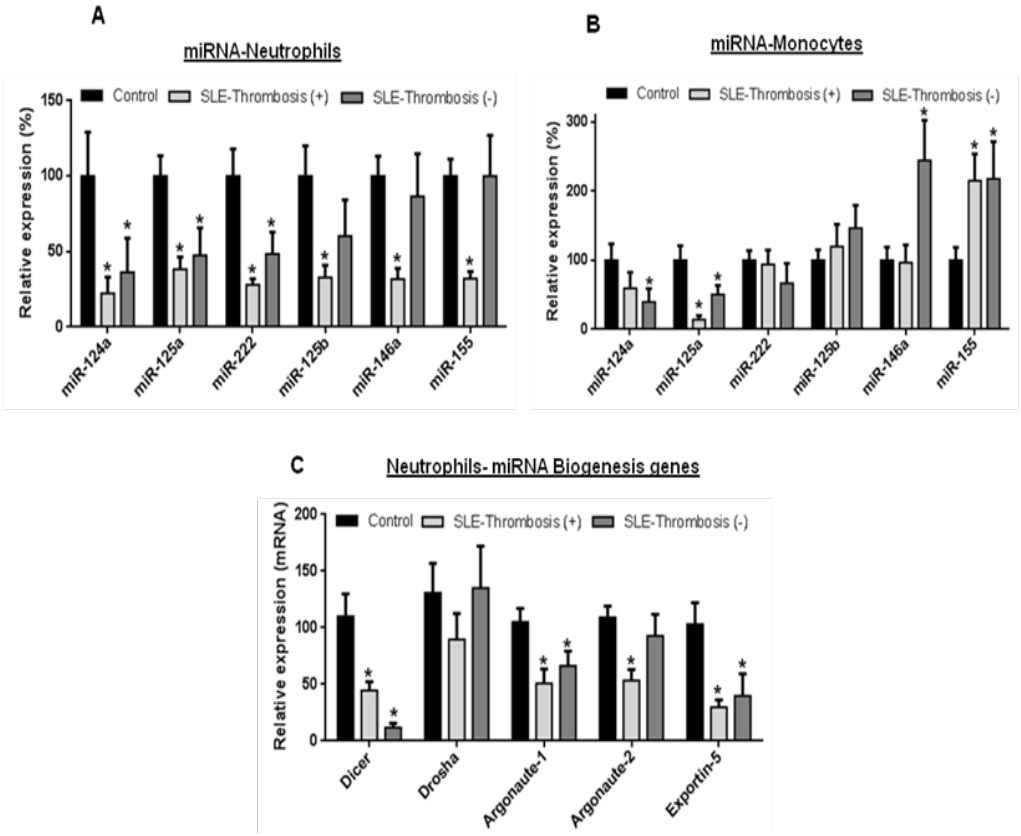
Supplementary Figure S3. Expression levels of the selected miRNAs in neutrophils and monocytes from patients with thrombosis, but without aPL. miRNA levels were measured in all the subjects included in the study (20 patients with previous venous or arterial thrombosis events and 20 healthy donors) on isolated neutrophils (A) or monocytes (B) by qRT-PCR and normalized with U6 snRNA. Differences were analyzed by Student's t test. Statiscal significance was taken as $p<0.05$.

Supplementary Figure S4



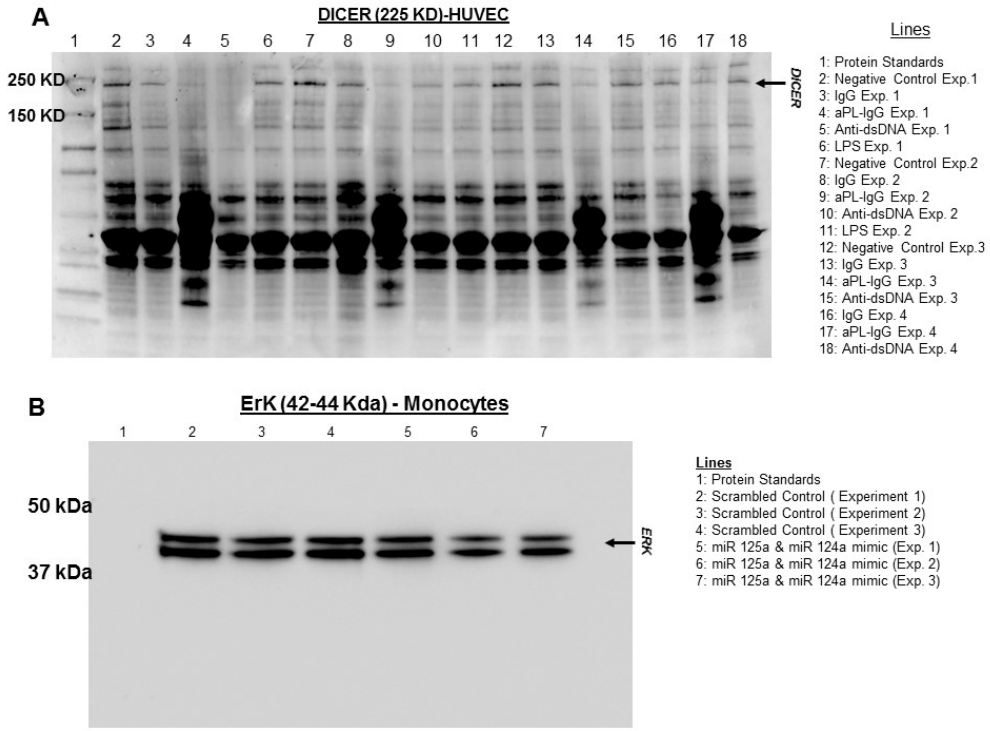
Supplementary Figure S4. In vitro effects of aPL-IgG and anti-dsDNA-IgG antibodies on the expression of proteins of biogenesis and inflammatory markers. Endothelial cells were treated in vitro with aPL-IgG antibodies purified from APS patients' serum, or with anti-dsDNA-IgG antibodies purified from SLE patients' serum, or with LPS, or with culture medium, or with synthetic human IgG. (A) Relative mRNA expression levels of miRNA biogenesis proteins, including Dicer, Drosha, Ago1, Ago2 and Exportin-5. Values are the mean and SEM from 4 independent experiments. Significant differences (*P<0.05) vs ECs treated with synthetic IgG. (B) Relative mRNA expression levels of various inflammatory mediators after the above described treatments. Values are the mean and SEM from 4 independent experiments. Significant differences (*P<0.05) vs ECs treated with synthetic IgG.

Supplementary Figure S5



Supplementary Figure S5. Expression levels of the selected miRNAs and biomarkers of miRNA-biogenesis in neutrophils and monocytes from SLE patients with thrombosis or without thrombosis in relation to healthy donors. (A) miRNA levels were measured in monocytes and neutrophils isolated from SLE patients with or without previous thrombotic events and in healthy donors by qRT-PCR and normalized with U6 snRNA. (B) Relative mRNA expression levels of miRNA biogenesis proteins, including Dicer, Drosha, Ago1, Ago2 and Exportin-5. Differences were analyzed by Student's t test. Statistical significance was taken as $p<0.05$.

Supplementary Figure S6



Supplementary Figure S6. Full-length blots for key data. (A) Western blot of samples from independent experiments showing Dicer expression after the treatments indicated in ECs. (B) Western blot of samples from 3 independent experiments showing the changes promoted in ERK expression by the simultaneous transfection of monocytes with miR-124a and miR-125a mimic

CONCLUSIONES

Conclusiones

1. Los anticuerpos anti-CCPs son mediadores clave en el desarrollo del perfil inflamatorio y proaterogénico de los pacientes con Artritis Reumatoide (AR), siendo sus efectos específicos de la diana celular sobre la que actúan. Por tanto, los anticuerpos anti-CCPs podrían ser considerados dianas terapéuticas para la prevención del desarrollo de enfermedad cardiovascular en pacientes con AR.

> *Anti-cyclic citrullinated protein antibodies are implicated in the development of cardiovascular disease in rheumatoid arthritis. Arteriosclerosis, Thrombosis, and Vascular Biology (ATVB). 2014*

2. El perfil de expresión génica permite la segregación del Síndrome Antifosfolípido Primario, el Lupus Eritematoso Sistémico y el Síndrome Antifosfolípido asociado a Lupus, con características específicas que explican los cambios pro-aterotrombóticos observados en estas enfermedades autoinmunes.

En su conjunto, los resultados de este estudio sugieren que la identificación de genes clave que regulan vías fisiopatológicas específicas permitiría el desarrollo de terapias dirigidas para cada condición autoimmune.

> *Gene profiling reveals specific molecular pathways in the pathogenesis of atherosclerosis and cardiovascular disease in Antiphospholipid syndrome, Systemic Lupus Erythematosus and Antiphospholipid Syndrome with Lupus. Annals of the Rheumatic Diseases* (*ARD*). *2015*

3. Diversos miRNAS, y sus proteínas de biogénesis se encuentran diferencialmente expresados en monocitos y neutrófilos de pacientes con Síndrome Antifosfolípido y Lupus Eritematoso Sistémico, y son modulados por acción de los anticuerpos característicos de ambas patologías. Dichos miRNAs podrían considerarse posibles biomarcadores de patología aterotrombótica en ambas enfermedades autoinmunes.

> *Atherothrombosis-associated microRNAs in Antiphospholipid syndrome and Systemic Lupus Erythematosus patients.* Scientific Reports *(*Sci. Rep.*). 2016*

4. En suma, los resultados de los estudios presentados indican que el análisis integrado de los procesos autoinmunes, moleculares, genéticos y epigenéticos, permiten la identificación y caracterización de nuevos biomarcadores de desarrollo de patología vascular aterotrombótica en enfermedades autoinmunes sistémicas.

CONCLUSIONS

Conclusions

1. Anti-CCPs are key players in the inflammatory and pro-atherogenic status of Rheumatoid Arthritis (RA) patients, showing specific effects on the immune cells targeted. Thus, targeting these autoantibodies would be an excellent strategy to prevent the development of cardiovascular disease in RA.

> *Anti-cyclic citrullinated protein antibodies are implicated in the development of cardiovascular disease in rheumatoid arthritis. Arteriosclerosis, Thrombosis, and Vascular Biology (ATVB). 2014*

2. Gene expression profiling allows the segregation of Antiphospholipid Syndrome, Systemic Lupus Erythematosus and Antiphospholipid Syndrome with Lupus, with specific signatures explaining the pro-atherosclerotic and pro-thrombotic alterations in these highly related autoimmune diseases.

The overall results of this study suggest that the identification of key genes that regulate specific pathophysiological pathways would allow the development of targeted therapies for each autoimmune condition.

> *Gene profiling reveals specific molecular pathways in the pathogenesis of atherosclerosis and cardiovascular disease in Antiphospholipid syndrome, Systemic Lupus Erythematosus and Antiphospholipid Syndrome with Lupus. Annals of the Rheumatic Diseases* (*ARD*). *2015*

3. Specific miRNAs and the proteins involved in their biogenesis are significantly altered in monocytes and neutrophils of Antiphospholipid Syndrome and Systemic Lupus Erythematosus patients by effect of the

specific antibodies of these disorders. In addition, altered miRNAs' expression is linked to autoimmunity, thrombosis, early atherosclerosis, and oxidative stress in both pathologies. Thus, these miRNAs could be considered potential biomarkers of atherothrombotic pathology in both autoimmune diseases.

> *Atherothrombosis-associated microRNAs in Antiphospholipid syndrome and Systemic Lupus Erythematosus patients.* Scientific Reports *(*Sci. Rep.*). 2016*

4. Taken together, the results of these studies indicate that the integrated analysis of the autoimmune, molecular, genetic and epigenetic processes allow the identification and characterization of new biomarkers for the development of atherothrombotic vascular pathology in systemic autoimmune diseases.

ANEXO

I- Informe del factor de impacto y cuartil del Journal citation Reports (SCI y/o SSCI) o de las bases de datos de referencia del área en el que se encuentran las publicaciones presentadas

Primer artículo:

Arterioscler Thromb Vasc Biol. 2014 Dec;34(12):2706-16. doi: 10.1161/ATVBAHA.114.304475.

Arteriosclerosis, Thrombosis, and Vascular Biology

Journal of the American Heart Association

American Heart Association.

Anticyclic Citrullinated Protein Antibodies Are Implicated in the Development of Cardiovascular Disease in Rheumatoid Arthritis

Nuria Barbarroja,* Carlos Pérez-Sanchez,* Patricia Ruiz-Limon, Carmen Castro-Villegas, Maria Angeles Aguirre, Rosario Carretero, Pedro Segui, Yolanda Jimenez-Gomez, Manuela Sanna, Antonio Rodriguez-Ariza, Eduardo Collantes-Estevez, Alejandro Escudero,* Chary López-Pedrera*

*These authors contributed equally to this article.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.114.304475

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Rank in Category: ARTERIOSCLEROSIS THROMBOSIS AND VASCULAR BIOLOGY

Journal Ranking

For **2014**, the journal **ARTERIOSCLEROSIS THROMBOSIS AND VASCULAR BIOLOGY** has an Impact Factor of **6.008**.

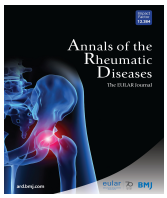
This table shows the ranking of this journal in its subject categories based on Impact Factor.

Category Name	Total Journals in Category	Journal Rank in Category	Quartile in Category
HEMATOLOGY	68	5	Q1
PERIPHERAL VASCULAR DISEASE	60	4	Q1

Segundo artículo:

Ann Rheum Dis. 2015 Jul;74(7):1441-9. doi: 10.1136/annrheumdis-2013-204600.

EXTENDED REPORT



Gene profiling reveals specific molecular pathways in the pathogenesis of atherosclerosis and cardiovascular disease in antiphospholipid syndrome, systemic lupus erythematosus and antiphospholipid syndrome with lupus

Carlos Perez-Sanchez,[1] Nuria Barbarroja,[1] Sebastiano Messineo,[2] Patricia Ruiz-Limon,[1] Antonio Rodriguez-Ariza,[1] Yolanda Jimenez-Gomez,[1] Munther A Khamashta,[3] Eduardo Collantes-Estevez,[1] Mª Jose Cuadrado,[3] Mª Angeles Aguirre,[1] Chary Lopez-Pedrera[1]

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Rank in Category: ANNALS OF THE RHEUMATIC DISEASES

Journal Ranking

For **2015**, the journal **ANNALS OF THE RHEUMATIC DISEASES** has an Impact Factor of **12.384**.

This table shows the ranking of this journal in its subject categories based on Impact Factor.

Category Name	Total Journals in Category	Journal Rank in Category	Quartile in Category
RHEUMATOLOGY	32	1	Q1

Tercer artículo:

Sci Rep. 2016 Aug 9;6:31375. doi: 10.1038/srep31375.

SCIENTIFIC REPORTS

OPEN

'Atherothrombosis-associated microRNAs in Antiphospholipid syndrome and Systemic Lupus Erythematosus patients'

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C. Pérez-Sánchez[1], M. A. Aguirre[1], P. Ruiz-Limón[1], N. Barbarroja[1], Y. Jiménez-Gómez[1], I. Arias de la Rosa[1], A. Rodriguez-Ariza[1], E. Collantes-Estévez[1], P. Segui[1], F. Velasco[1], M. J. Cuadrado[2], R. Teruel[3], R. González-Conejero[3], C. Martínez[3] & Ch. López-Pedrera[1]

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Journal Ranking

For **2015**, the journal **Scientific Reports** has an Impact Factor of **5.228**.

This table shows the ranking of this journal in its subject categories based on Impact Factor.

Category Name	Total Journals in Category	Journal Rank in Category	Quartile in Category
MULTIDISCIPLINARY SCIENCES	63	7	Q1

II- **Producción Cinetífica**

1. Arroyo AB, Salloum-Asfar S, PÉREZ-SÁNCHEZ C, Teruel-Montoya R, Navarro S , García-Barberá N , Luengo-Gil G , Roldán V, Hansen JB , López-Pedrera Ch, Vicente V, González-Conejero1 R, Martínez C. Regulation of TFPIα expression by miR-27a/b-3p in human endothelial cells under normal conditions and in response to androgens. Sci Rep. 2017 Jan. In Press. IF:5.22. Q1.

2. Ruiz-Limón P, Ortega R, Arias de la Rosa I, Abalos-Aguilera MC, PÉREZ-SÁNCHEZ C, Jiménez-Gómez Y, Peralbo-Santaella E, Font P, Ruiz-Vilchez D, Ferrin G, Collantes-Estevez E, Escudero A, López-Pedrera Ch and Barbarroja N. Tocilizumab improves the pro-atherothrombotic profile of rheumatoid arthritis patients modulating endothelial dysfunction, NETosis and inflammation. Transl Res. 2016 Dec 9. Pii: S1931-5244(16)30423-6. IF:4,55. Q1(D1).

3. Ruiz-Limon P, Barbarroja N, PEREZ-SANCHEZ C, Aguirre MA, Bertolaccini ML, Khamashta MA, Rodriguez-Ariza A, Almadén Y, Segui P, Khraiwesh H, Gonzalez-Reyes JA, Villalba JM, Collantes-Estevez E, Cuadrado MJ, Lopez-Pedrera C. Atherosclerosis and cardiovascular disease in systemic lupus erythematosus: effects of in vivo statin treatment. Ann Rheum Dis. 2015 Jul;74(7):1450-8. IF:12.3. Q1(D1).

4. Castro-Villegas C, PÉREZ SÁNCHEZ C*, Escudero A, Filipescu I, Verdu M, Ruiz-Limón P, Aguirre MA, Jiménez-Gomez Y, Font P, Rodriguez-Ariza A, Peinado JR, Collantes-Estévez E, González-Conejero R, Martinez C, Barbarroja N, López-Pedrera Ch. Circulating miRNAs as potential biomarkers of therapy effectiveness in Rheumatoid Arthritis patients treated with anti-TNFα. Arthritis Res Ther. 2015 Mar. 9;17:49. IF: 4.117. Q1. *SHARED FIRST AUTHORSHIP.

5. Herencia C, Almadén Y, Martínez-Moreno JM, Espejo I, Herrera C, PÉREZ-SÁNCHEZ C, Guerrero F, Ciria R, Briceño FJ, Ferrín G, de la Mata M, Muñoz-Castañeda JR. Human mesenchymal stromal cell lysates as a novel strategy to recover liver function. Regen Med. 2015;10(1):25-38. IF: 2,602. Q2.

6. López-Pedrera Ch, Aguirre MA, Ruiz-Limon P, PÉREZ-SÁNCHEZ C, Jimenez-Gomez Y, Barbarroja , Cuadrado MJ. Immunotherapy in antiphospholipid síndrome. Int Immunopharmacol. 2015 Aug;27(2):200-8. IF:2,55. Q2

7. PEREZ-SANCHEZ C, Ruiz-Limon P, Aguirre MA, Bertolaccini ML, Khamashta MA, Rodriguez-Ariza A, Segui P, Collantes-Estevez E, Barbarroja N, Khraiwesh H, Gonzalez-Reyes JA, Villalba JM, Velasco F, Cuadrado MJ, Lopez-Pedrera C. Mitochondrial dysfunction in antiphospholipid syndrome: implications in the pathogenesis of the disease and effects of coenzyme Q(10) treatment. Blood. 2012 Jun 14;119(24):5859-70. IF:9,06. Q1(D1).

8. López-Pedrera C, PEREZ-SANCHEZ C, Ramos-Casals M, Santos-Gonzalez M, Rodriguez-Ariza A, Cuadrado MJ. Cardiovascular risk in systemic autoimmune diseases: epigenetic mec hanisms of immune-regulatory functions. Clin Dev Immunol. 2012;2012:974648. IF:3.064. Q2

9. Teruel R, PEREZ-SANCHEZ C, Corral J, Herranz MT, Pérez-Andreu V, Saiz E, García-Barberá N, Lozano J, Martínez-Martínez I, Roldán V, Vicente V, López-Pedrera C, Martínez C. Identification of miR-19b and miR-20a as new modulators of tissue factor expression in patients with systemic lupus erythematosus and antiphospholipid syndrome. Journal of Thrombosis and Haemostasis. 2011 Jun, 22. J Thromb Haemost. 2011 Oct;9(10):1985-92. IF: 5.731. Q1(D1).

10. López- Pedrera C, Ruiz-Limón P, Aguirre MA, Barbarroja N, PÉREZ-SÁNCHEZ C, Buendía P, Rodriguez-García IC, Rodriguez-Ariza, Collantes-Estevez E, Velasco F, Khasmahta M, Cuadrado MJ. Global effects of fluvastatin on the prothrombotic status of patients with antiphospholipid syndrome. Ann Rheum Dis. 2011 Apr;70(4):675-82. IF:8.71. Q1(D1)

11. Barbarroja N, Torres LA, Rodriguez-Ariza A, Valverde-Estepa A, Lopez-Sanchez LM, Ruiz-Limón P, PÉREZ-SÁNCHEZ C, Carretero RM, Velasco F, López-Pedrera Ch. AEE788 is a vascular endothelial growth factor receptor tyrosine kinase inhibitor with antiproliferative and proapoptotic effects in Acute Myeloid Leukemia. Exp Hematol. 2010 Aug;38(8):641-52 IF: 3.198. Q2.

III- Patentes

1. Título propiedad industrial registrada: MiRNAs circulantes como biomarcadores del Síndrome Antifosfolípido Primario.
Inventores/autores/obtentores: Rosario López Pedrera; María Ángeles Aguirre Zamorano; Eduardo Collantes Estévez; Nuria Barbarroja Puerto; Carlos Pérez Sánchez; Yolanda Jiménez Gómez.
Entidad titular de derechos: Servicio Andaluz de Salud, Universidad de Córdoba.
Nº de solicitud: P201630725.
País de inscripción: España, Andalucía.
Fecha de registro: 01/06/2016.

2. Título propiedad industrial registrada: MiRNAs como biomarcadores para el diagnóstico de cáncer de pulmón.
Inventores/autores/obtentores: Antonio Rodríguez Ariza, Rosario López Pedrera; Nuria Barbarroja Puerto; Carlos Pérez Sánchez.
Entidad titular de derechos: Servicio Andaluz de Salud, Universidad de Córdoba.
Nº de solicitud: P201630628.
País de inscripción: España, Andalucía.
Fecha de registro: 13/03/2016.

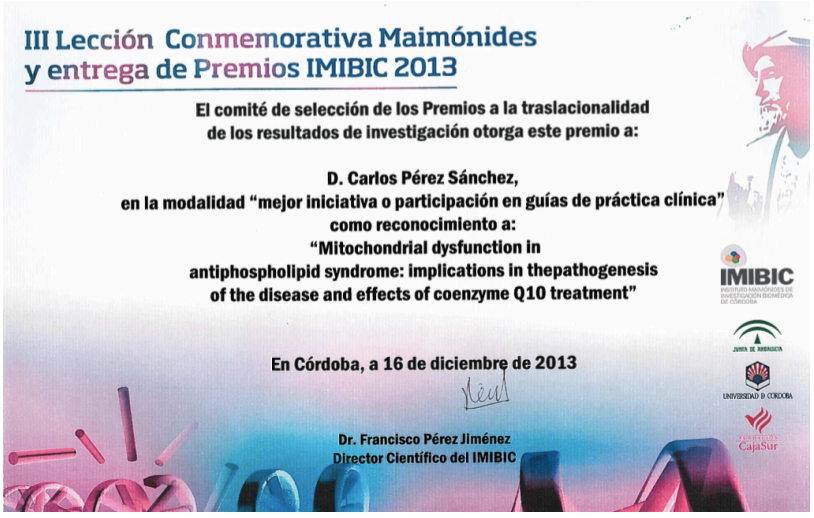
3. Título propiedad industrial registrada: MiRNAs circulantes como biomarcadores de efectividad terapéutica en pacientes con Artritis Reumatoide tratados con anti-TNFalpha.
Inventores/autores/obtentores: Rosario López Pedrera; Carlos Pérez Sánchez; Carmen Castro Villegas; Eduardo Collantes Estévez; Nuria Barbarroja Puerto; Patricia Ruiz Limón; Yolanda Jiménez Gómez
Entidad titular de derechos: Fundación Progreso y Salud.
Nº de solicitud: EP14382178.3.
País de inscripción: España, Andalucía.
Fecha de registro: 22/05/2014.

IV-Premios

Descripción: Premio a la publicación científica más relevante en colaboración con grupos internacionales
Fechas: 15 de Diciembre de 2015
Universidad o Entidad: IMIBIC
País: España
Observaciones: V Lección Conmemorativa Maimónides y Premios IMIBIC 2015

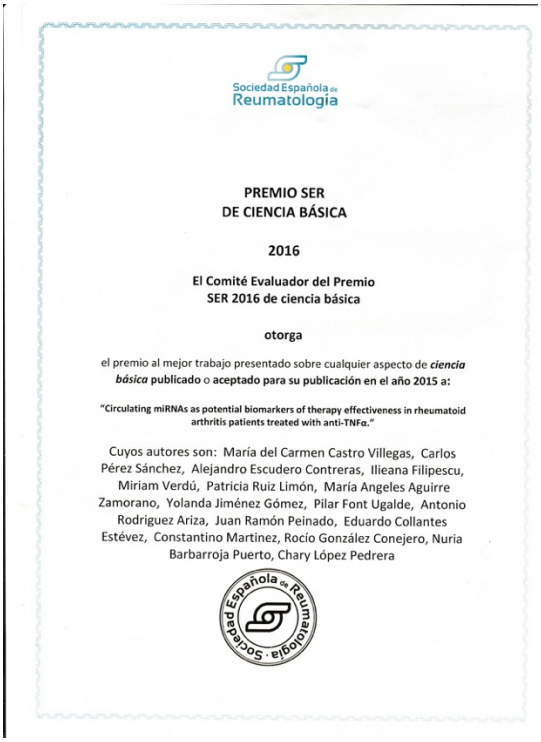
Descripción: Premio a la mejor comunicación oral
Fechas: 6 de Mayo de 2014
Universidad o Entidad: IMIBIC
País: España
Observaciones: V Jornada de jóvenes Investigadores del IMIBIC

Descripción: Premio a la mejor iniciativa o participación en guías de práctica clínica
Fechas: 16 de Diciembre de 2013
Universidad o Entidad: IMIBIC
País: España
Observaciones: III Lección Conmemorativa Maimónides y Premios IMIBIC 2013



Descripción: XI Premio Nacional de Investigación
Fechas: 11 de Diciembre de 2013
Universidad o Entidad: Fundación Ilustre Colegio de Médicos de Córdoba
País: España
Observaciones: Patrocinado por Fundación Barclays

Descripción: Premio SER 2016 de Ciencia Básica
Fechas: Mayo de 2016
Universidad o Entidad: Sociedad Española de Reumatología
País: España



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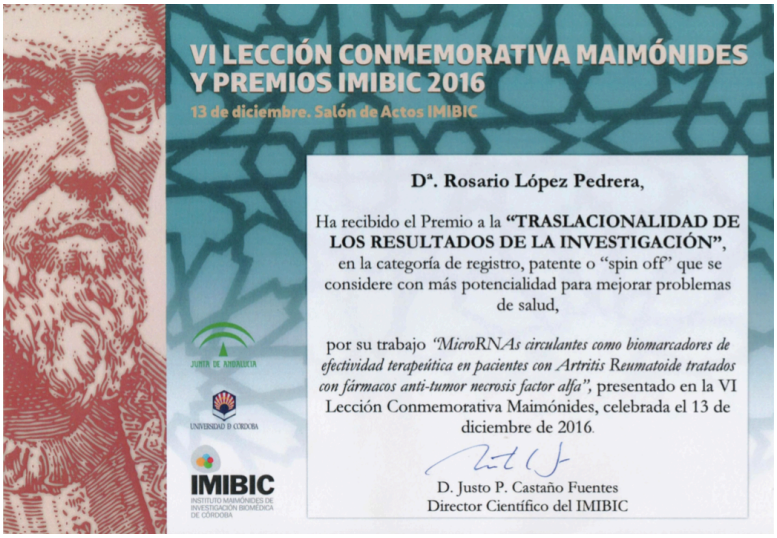
el premio al mejor trabajo presentado sobre cualquier aspecto de ***ciencia básica*** **publicado o aceptado para su publicación en el año 2015 a:**

"Circulating miRNAs as potential biomarkers of therapy effectiveness in rheumatoid arthritis patients treated with anti-TNFα."

Cuyos autores son: María del Carmen Castro Villegas, Carlos Pérez Sánchez, Alejandro Escudero Contreras, Ilieana Filipescu, Miriam Verdú, Patricia Ruiz Limón, María Angeles Aguirre Zamorano, Yolanda Jiménez Gómez, Pilar Font Ugalde, Antonio Rodríguez Ariza, Juan Ramón Peinado, Eduardo Collantes Estévez, Constantino Martínez, Rocío González Conejero, Nuria Barbarroja Puerto, Chary López Pedrera

Sociedad Española de Reumatología

Descripción: Premio a la traslacionalidad de los resultados de la investigación
Fechas: 13 de Diciembre de 2016
Universidad o Entidad: IMIBIC
País: España
Observaciones: VI Lección Conmemorativa Maimónides y Premios IMIBIC 2016



VI LECCIÓN CONMEMORATIVA MAIMÓNIDES Y PREMIOS IMIBIC 2016

13 de diciembre. Salón de Actos IMIBIC

Dª. Rosario López Pedrera,

Ha recibido el Premio a la **"TRASLACIONALIDAD DE LOS RESULTADOS DE LA INVESTIGACIÓN"**, en la categoría de registro, patente o "spin off" que se considere con más potencialidad para mejorar problemas de salud,

por su trabajo *"MicroRNAs circulantes como biomarcadores de efectividad terapeútica en pacientes con Artritis Reumatoide tratados con fármacos anti-tumor necrosis factor alfa"*, presentado en la VI Lección Conmemorativa Maimónides, celebrada el 13 de diciembre de 2016.

D. Justo P. Castaño Fuentes
Director Científico del IMIBIC

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